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(Direktor Prof. Dr. med. Bernhard J. Hering)

der University of Minnesota, Minneapolis, USA

PRETRANSPLANT TOLERANCE INDUCTION REDUCES THE ISLET MASS REQUIRED TO REVERSE DIABETES IN NOD MICE

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DEKAN:	**Prof. Dr. rer. nat. Heyo K. Kroemer**
1. Gutachter:	**Prof. Dr. med. Ch. Schütt (Greifswald)**
2. Gutachter:	**Prof. Dr. med. B. Hering (Minneapolis)**
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PRETRANSPLANT TOLERANCE INDUCTION REDUCES THE ISLET MASS REQUIRED TO REVERSE DIABETES IN NOD MICE

LIST OF ABBREVIATIONS

2-ME	2-mercaptoethanol
AEC	3-amino-9-ethylcarbazole
ALS	anti-mouse lymphocyte serum
BMT	bone marrow transplantation
BSA	bovine serum albumin
BW	body weight
CD	cluster of differentiation
CNI	calcineurin inhibitors
CsA	cyclosporine-A
e.g.	exempli gratia = for example
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GVHD	graft versus host disease
HBSS	Hank's balanced salt solution
H&E	hematoxylin and eosin
i.e.	id est = that means
IFN-γ	interferon-gamma
i.p.	intraperitoneal
IPGTT	intraperitoneal glucose tolerance test
M	Molar
mg/kg/d	mg/kg per day
MR1	anti-CD154 (anti-CD40ligand) monoclonal antibody
n/a	not available

NaCl	sodium chloride
NO	nitric oxide
NOD	non-obese diabetic
PBS	phosphate buffered saline
PE	phycoerythrin
PE-50	polyethylene 50 tubing
PEC	resident peritoneal cells
POD	postoperative day
RPMI-1640	Roswell Park Memorial Institute culture medium Number 1640
s.c.	subcutaneous
SRL	sirolimus
STZ	streptozotocin
TGF-ß	transforming growth factor-beta
Th1 / 2	T helper cell type 1 / 2
vs.	versus

Introduction

Type 1 diabetes mellitus is a chronic autoimmune disease that results in most cases from a T cell-regulated destruction of the insulin-producing pancreatic beta-cells in the islets of Langerhans (1-4). It accounts for about 5-10% of all diabetes cases and the worldwide incidence of this disease ranges between 0.57 and more than 40 per 100,000 per year depending on varying genetic susceptibility in different racial populations and environmental factors (1; 2). An autoimmune disease develops when the humoral and cellular immune systems fail to distinguish self from non-self. It is thought, that in genetically susceptible individuals, type 1 diabetes mellitus can be initiated during a viral infection, when viral proteins share an amino acid sequence with a beta-cell protein, e.g. glutamic acid decarboxylase, that lead to self-reactive T cell clones (2). This process is known as molecular mimicry. Alternatively, an infection with a beta-cell-tropic virus, like Coxsackie strain B4, could lead to an increased local cytokine release, resulting in the activation of cytotoxic T cells as well as B cells, augmentation of the local inflammatory response and consecutively to islet cell loss (2; 5). Treatment of choice for type 1 diabetes mellitus is exogenous insulin administration accompanied with glucose self-monitoring and nutritional planning. Since prolonged exposure to hyperglycemia in diabetic patients can lead to neurological, micro- and macrovascular long-term complications (6), near physiologic control of glucose levels is the goal in the management of the disease (7). However, intensive insulin treatment often cannot fully achieve this target (1), and some diabetics experience severe hypoglycemic events and a reduced quality of life (8).

A different approach to the treatment of diabetes is pancreas transplantation that was first performed by Kelly, Lillehei and co-workers at the University of Minnesota in the late 1960s (9). Over the years the surgical procedures, graft preservation and

outcomes have greatly improved (10). However, due to the risks associated with this major operation as well as the immunosuppression, this treatment remains mostly available for diabetic patients with end-stage renal disease. In such a setting, combined kidney-pancreas transplantation has advantages over kidney transplantation alone (11).

A promising treatment option is the transplantation of pancreatic islets of Langerhans that can lead to restoration of normoglycemia and insulin independence after only minimally invasive surgery or with a radiological percutaneous method and ultrasound guidance (12). After harvesting a donor pancreas, islets of Langerhans can be extracted by collagenase digestion followed by density centrifugation using an automated technique (13; 14). In the clinical setting, islets are commonly delivered into the portal vein (Figure 1) and, following their embolization in the liver, they form a new blood supply (12). Since the first islet transplantation in rodent models in 1972 (15), techniques of islet isolation, transplantation and peri-transplant management have evolved tremendously (12). In 2000, Shapiro and co-workers published their groundbreaking series of islet transplants using a new glucocorticoid-free immunosuppression protocol, the ‘Edmonton Protocol’ (16). The group reported that all diabetic patients treated with this protocol received islets isolated from two to four donor pancreases and achieved normal blood glucose control and insulin independence out to 14 months (16). This report was followed by others transplanting islets isolated from two to three pancreases (17-21), from a single donor pancreas (22; 23), and, in a first recipient, from a living donor hemipancreas (24). Currently, the insulin independence rate is approximately 70% at one-year after islet transplantation, but long-term results still need to improve (12). For islet transplantation to become a more available and affordable treatment option, diabetes

reversal must be achieved and maintained, as with pancreas transplants, with a single donor pancreas on a consistent basis. Furthermore, the significant risks of the long-term side effects of immunosuppressive drugs (25) limit the use of current transplant protocols to diabetic patients with severe treatment difficulties, like hypoglycemia unawareness (12).

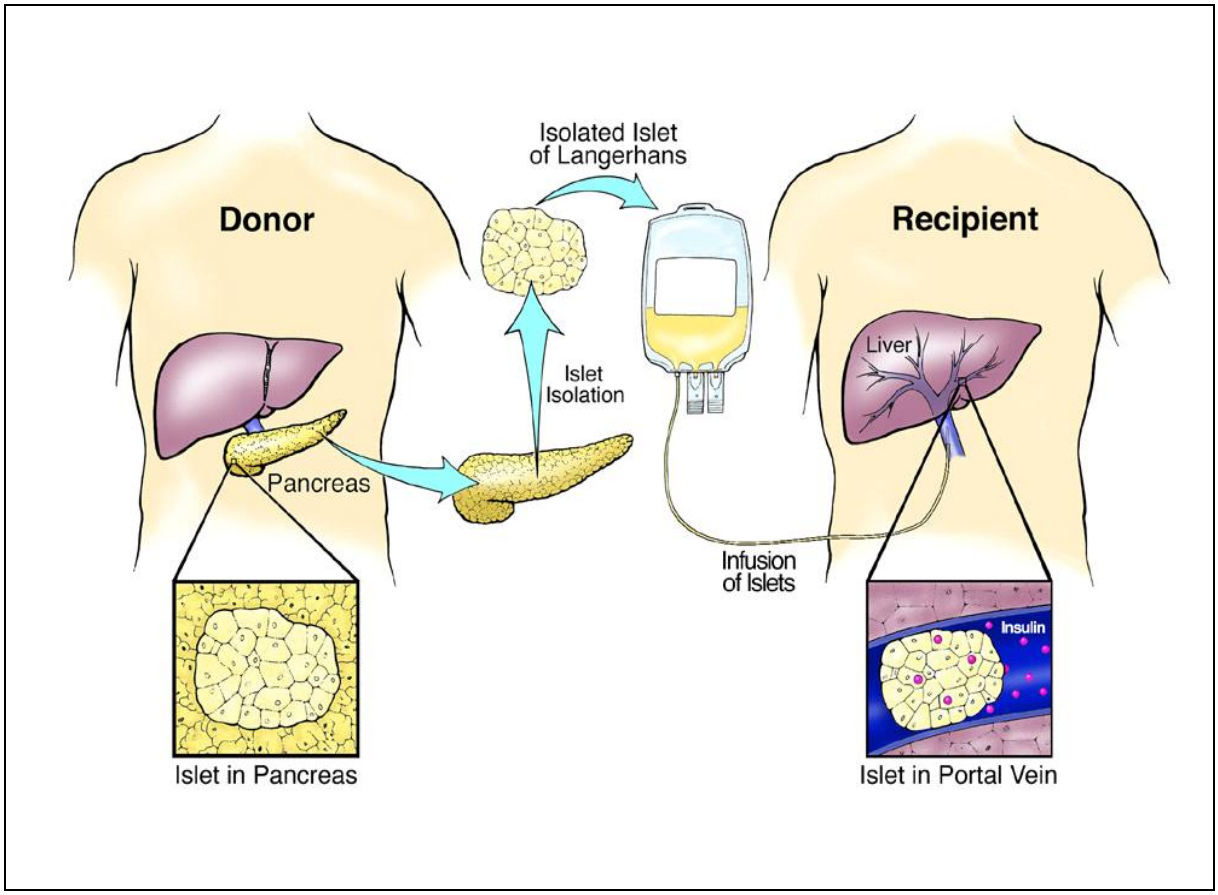

Figure 1. Systematic overview of the islet transplantation procedure in the clinical setting. After harvesting a donor pancreas, islets can be extracted by collagenase digestion followed by density gradient separation. The transplantation of islets is performed by portal vein infusion using radiological guidance or a surgical procedure. Figure by B.J. Hering, Diabetes Institute for Immunology and Transplantation.

Tolerance induction to islet allografts has the potential to overcome both problems: The requirement for chronic immunosuppression and the need for multiple donors. It has been shown that islet autotransplant recipients require a much lower number of islets than recipients in the 'Edmonton Protocol' to establish insulin independence

(26-30). Thus, the presence of tolerance towards an islet allograft might reduce the number of islets needed to reverse diabetes, due to the elimination of allo- and autoreactivity in addition to the elimination of drug toxicity towards the graft. It is known that the existence of autoimmunity seems to be very resistant towards commonly used immunosuppression (31-33), and reported findings on the roles of adaptive immunity and immunosuppression on islet engraftment seem to be conflicting (34-36).

After the pioneering work of Medawar, Owen and others, beginning more than 50 years ago, researchers have known that hematopoietic chimerism can be associated with donor-specific tolerance (37-39). Mixed hematopoietic chimerism refers to a state in which donor and host hematopoietic cell lineages coexist in the recipient and, in contrast to full chimerism, mixed chimeras retain a superior immunocompetence (40). Due to intrathymic deletion of donor- and host-reactive T cells in addition to peripheral tolerance mechanisms that are not yet fully understood, the recipient is tolerant towards the bone marrow donor while normal immune responses to third-party antigens are preserved (41-43). Mixed hematopoietic chimerism represents an attractive candidate for clinical use in organ transplantation and can be induced by transplantation of hematopoietic stem cells into an appropriately conditioned host. In the setting of islet transplantation, it appears that low levels of stable donor chimerism may be sufficient to induce transplant tolerance and control autoreactivity (44-47). In experimental studies, mixed chimerism can be achieved with nonmyeloablative regimens of minimal toxicity using costimulatory blockade in conjunction with bone marrow transplantation (48-51).

In this study, the non-obese diabetic (NOD) mouse model was used, since it represents an interesting model of type 1 diabetes mellitus with spontaneous diabetes development based on autoimmune mechanisms (52; 53). Mixed hematopoietic chimerism was induced with an irradiation-free, nonmyeloablative regimen and costimulatory blockade of CD40ligand. After islet transplantation, diabetes reversal rates were compared with NOD mice treated similar to the 'Edmonton protocol' with polyclonal T cell antibodies, tacrolimus and sirolimus (SRL).

Materials and Methods

Animals

Female NOD mice were obtained from Jackson Laboratories (Bar Harbor, ME). Starting at the age of 12 weeks, mice were screened weekly for diabetes by tail vein glucose measurements. As soon as hyperglycemia was present, mice were treated with daily subcutaneous (s.c.) injections of human NPH insulin (Novo Nordisk Pharmaceuticals, Princeton, NJ) until postoperative day (POD) -1 (immunosuppressed mice) or POD -22 (bone marrow recipients). Animals receiving bone marrow were implanted with insulin pellets (LinShin Inc., Scarborough, ON, Canada) on POD -20 and pellets were removed on POD -1. All mice were diabetic with blood glucose levels greater than 400 mg/dl for at least two weeks before they received bone marrow or immunosuppressive drugs. Male Balb/c, C3H and C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) or Taconic Farms (Germantown, NY).

Donor and recipient mice were housed in microisolator cages under specific pathogen-free conditions and were given standard food pellets and water *ad libitum*. Animals receiving bone marrow were given autoclaved food and water containing sulfamethoxazole and trimethoprim (Qualitest Pharmaceuticals, Inc., Huntsville, AL) and their cages were autoclaved for the following three weeks. All experiments were performed according to the protocols reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee.

Experimental Groups

The following study groups were established (Table 1): In Group 1 (n=31) NOD mice received conditioning therapy, including fludarabin phosphate (Fludara, 400 mg/kg,

Berlex Laboratories, Wayne, NJ) and cyclophosphamide (Cytoxan, 200 mg/kg, Bristol-Myers Squibb Co, Princeton, NJ), which were both given intraperitoneally (i.p.) on POD –22. In addition, anti-CD154 monoclonal antibody (MR1, 0.4 mg i.p., Bioexpress, West Lebanon, NH) was administered daily from POD -21 to -17, then on POD -15, -12 and -8, similar to previously described (17). Bone marrow cells were transfused (BMT) on POD -21. 100 to 400 Balb/c islets were transplanted on POD 0. Group 2 (n=36) recipients received tacrolimus (Prograf, 0.5 mg/kg/day s.c., Fujisawa Healthcare, Deerfield, IL) and SRL (Rapamune, 1 mg/kg/day orally by gavage, Wyeth Laboratories, Philadelphia, PA) daily from POD –2. Rabbit anti-mouse lymphocyte serum (ALS, 0.3 ml i.p., Accurate Chemical and Scientific Corporation, Westbury, NY) was given on POD –1 and 0. In Group 3 (n=7), C57BL/6 mice received a single i.p. injection of 220 mg/kg streptozotocin (STZ, Sigma, St. Louis, MO) between POD -20 and -10 before being transplanted with a low-dose (75 and 100 islets) syngeneic islet graft on POD 0. Starting on POD 60 or 100, these mice were immunosuppressed with the same dose and schedule of tacrolimus and SRL as Group 2 recipients. In Group 4 (n=5), NOD mice received an islet transplant of 100 islets after conditioning therapy and BMT. In addition, these mice were administered tacrolimus and SRL at the same dose and schedule as Group 2 starting on POD -2. Group 5 (n=6) animals were given the same treatment as Group 1 animals; on POD 250 these animals underwent graft nephrectomy and, after return to hyperglycemia, were retransplanted with 100 or 200 Balb/c islets to the right kidney capsule. In Group 6 (n=2), conditioning therapy and BMT were followed by third-party C3H islet transplants on POD 0. Group 7 included untreated diabetic NOD mice that received Balb/c islets (n=6). Animals in Groups 8 (n=9) and 9 (n=5) received the same treatment as Group 1 and 2 mice, respectively. Following the transplantation of 200 islets on POD 0, resident peritoneal macrophages were harvested on POD 1.

In Groups 1, 4, 5 and 6 islet transplants were only performed in animals with chimerism levels >40%. Group 8 mice had chimerism levels >20%.

Table 1. Treatment Groups

Group	Number of Animals	Islet Donor	Recipient	Recipient Treatment	Days to Normoglycemia	Normoglycemia at POD 30	Histological signs of rejection at POD 30
1	31	Balb/c	NOD	Fludara 400 mg/kg, cyclophosphamide 200 mg/kg, MR1 0.4 mg x8, BMT	100 islets: 2,3,10,19,19, >30 200 islets: 1,1,1,1,5,6,6,6,9 300 islets: 1,1,1,1,1,1,1,6 400 islets: 1,1,1,1,2,3,4,7	100 islets: 5/6 200 islets: 9/9 300 islets: 8/8 400 islets: 8/8	100 islets: 0/6 200 islets: 0/9 300 islets: 0/8 400 islets: 0/8
2	36	Balb/c	NOD	ALS 0.3 ml x2, SRL 1 mg/kg/d, tacrolimus 0.5 mg/kg/d	100 islets: >30,>30,>30,>30 200 islets: 1,1, >30 x10 300 islets: 1,1,1,1,1,1,>30x6 400 islets: 1,1,1,1,1,6,>30x2	100 islets: 0/4 200 islets: 2/12 300 islets: 6/12 400 islets: 6/8	100 islets: 0/4 200 islets: 0/12 300 islets: 0/12 400 islets: 0/8
3	7	C57BL/6	C57BL/6	STZ, stable islet grafts, SRL 1 mg/kg/d, tacrolimus 0.5 mg/kg/d	n/a	n/a	n/a
4	5	Balb/c	NOD	Fludara 400 mg/kg, cyclophosphamide 200 mg/kg, MR1 0.4 mg x8, BMT, SRL 1 mg/kg/d, tacrolimus 0.5 mg/kg/d	100 islets: 2, >30x4	100 islets: 1/5	100 islets: 0/5
5	6	Balb/c	NOD	Fludara 400 mg/kg, cyclophosphamide 200 mg/kg, MR1 0.4 mg x8, BMT, islet retransplantation on POD 250	100 islets: 4,5,9 200 islets: 2,3,3	100 islets: 3/3 200 islets: 3/3	n/a n/a
6	2	C3H	NOD	Fludara 400 mg/kg, cyclophosphamide 200 mg/kg, MR1 0.4 mg x8, Balb/c BMT	400 islets: 2,2	400 islets: 0/2	400 islets: 2/2
7	6	Balb/c	NOD	-	400 islets: 1,1,1,2,2,3	400 islets: 0/6	400 islets: 6/6
8	9	Balb/c	NOD	Fludara 400 mg/kg, cyclophosphamide 200 mg/kg, MR1 0.4 mg x8, BMT	n/a	n/a	n/a
9	5	Balb/c	NOD	ALS 0.3 ml x2, SRL 1 mg/kg/d, tacrolimus 0.5 mg/kg/d	n/a	n/a	n/a

Bone Marrow Transplantation

Femoral and tibial bones from 4- to 6-week-old male Balb/c mice were removed and the marrow was flushed out with Dulbecco's PBS (phosphate buffered saline, Gibco, Grand Island, NY) containing 2 µl/ml gentamicin (Elkins-Sinn, Inc., Cherry Hill, NJ) using a 10 ml syringe with a 26-gauge needle. Bone marrow cell suspensions were washed twice with PBS and a total of $8x10^7$ viable cells were transfused via the tail vein.

Determination of Chimerism Levels

To determine donor chimerism levels, blood was collected on POD -3 via sinus orbitalis puncture and heparinized. Cells were incubated on ice for 30 minutes with 1 µg anti-H-$2D^d$, anti-H2-D^b, anti-CD3 and anti-CD45R/B220 primary antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE) or CyChrome (all from BD PharMingen, SanDiego, CA). Red blood cells were lysed (PharmLyse, PharMingen, San Diego, CA) and thereafter washed in PBS and 2% fetal calf serum. Stained cells were measured in a fluorescence-activated cell sorter (FACScan, Becton Dickinson, Mountain View, CA) and analyzed with Cellquest software (Becton Dickinson, Mountain View, CA). Lymphocyte, granulocyte and monocyte populations were gated for multilineage analysis and two-color flow cytometric analysis was used to distinguish between donor and host cells. Percentage of mixed chimerism was calculated by dividing the net percentage of donor cells by the total net percentage of donor plus host cells of that lineage.

Islet Isolation and Transplantation

Pancreatic islets were isolated and transplanted as previously described (54; 55). 2.5 ml of Hank's balanced salt solution (HBSS, Life Technology, Gaithersburg, MD)

containing 2 mg/ml collagenase from clostridium histolyticum (Serva, Heidelberg, Germany) was injected into the pancreatic duct. The distended pancreas was removed and dissociated at 37 ºC for 16 minutes. Islets were purified by centrifugation on gradients comprising three different densities (1.130, 1.110 and 1.070 g/cm^3, OptiPrep^{TM}, Accurate Chemical, Westbury, NY). Thereafter, the islet layer was collected, the islets washed with Medium E199 Solution (Mediatech Cellgro, Herndon, VA) and then handpicked and counted. Only islets measuring 150 to 250 µm in diameter, free of acinar cells, vessels, lymph nodes and ducts, were used for transplantation (55; 56). Recipient mice were anesthetized with tribomoethanol (Avertin, 0.0120 ml/g i.p.) and the left kidney was exposed through a lumbar incision (Figure 2a). PE-50 polyethylene tubing (Becton Dickinson, Parsippany, NJ) containing 100, 200, 300 or 400 islets was inserted beneath the kidney capsule at the lower pole and gently pushed to the upper one (Figure 2b). The islets were seeded at the upper pole (Figure 2c) by aid of an attached Hamilton syringe (Hamilton Company, Reno, NV). The capsular incision was then sealed with a cautery loop (Aaron Medical Industry, St. Petersburg, FL) and the animal was closed.

Assessment of Graft Function

In each recipient, non-fasting blood glucose levels and body weight was measured daily from POD 0 to 7 and three times a week thereafter until the end of the experiment. Restoration of stable normolycemia was defined as permanent reduction of elevated blood glucose levels <200 mg/dl before POD 21 and maintenance of normoglycemia through POD 30. The first day of stable normoglycemia was recorded as the first of five consecutive days of normoglycemia. Grafts that resulted in only temporary normoglycemia were considered unsuccessful.

All recipients underwent islet graft nephrectomy on POD 30 (or POD 250 and 280 in Group 5) to confirm that normoglycemia, if present, was due to the islet graft and to procure tissue for graft histology.

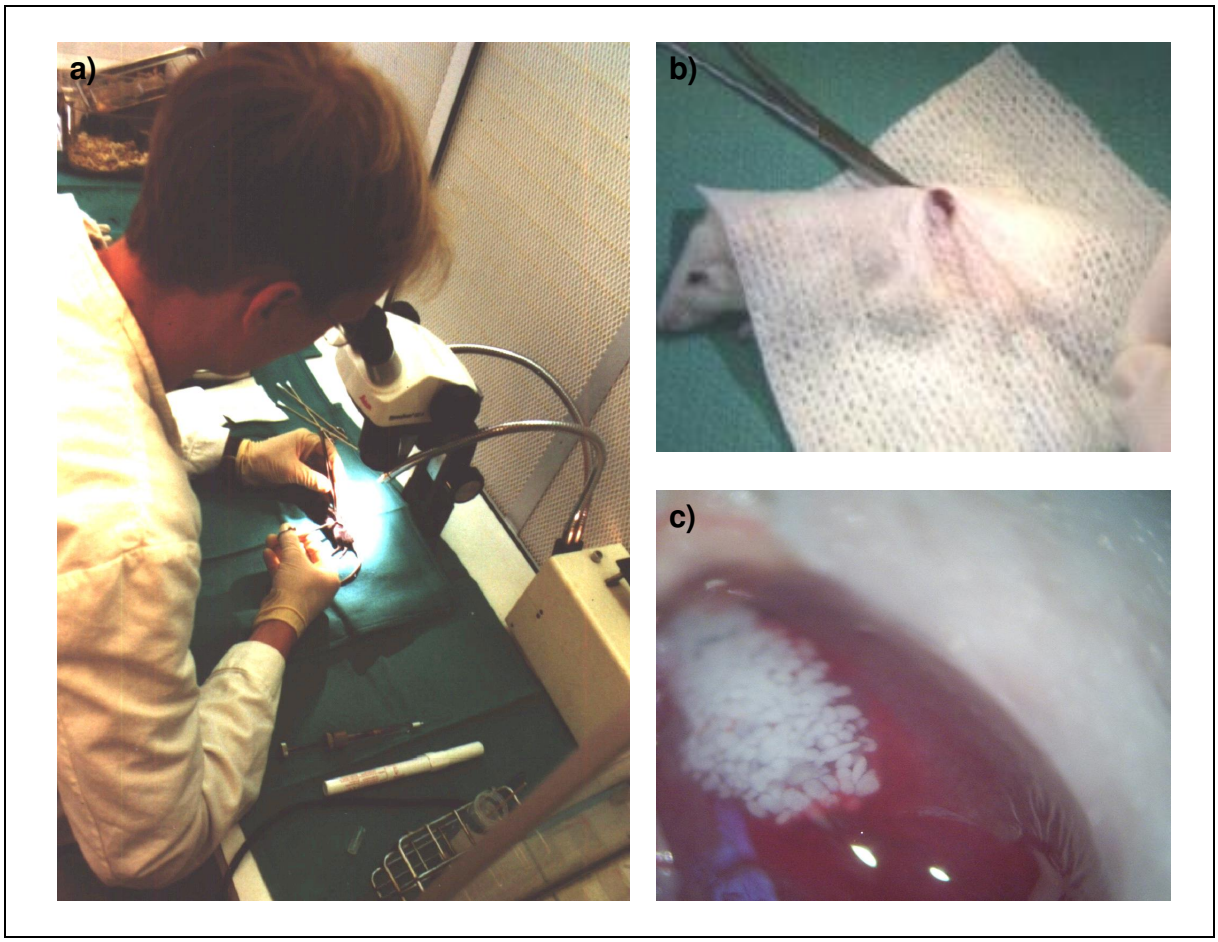

Figure 2. Transplantation of pancreatic islets in the mouse model. In anesthetized mice, the left kidney was exposed through a lumbar incision (a) and PE-50 tubing containing islets was inserted beneath the kidney capsule (b). 100 to 400 islets were seeded at the upper pole (c) by aid of a Hamilton syringe and the capsular incision was thereafter sealed. Pictures by Hannes Kalscheuer.

Intraperitoneal Glucose Tolerance Test

An intraperitoneal glucose tolerance test (IPGTT) was performed in fasted mice with reversed diabetes on POD 30. Following infusion of glucose (1 g/kg/BW, i.p., in 0.3 ml NaCl), blood glucose was measured at 0, 10, 20, 30 and 60 minutes.

Graft Histology and Immunohistochemistry

The examination of the islet-bearing kidney was performed on POD 30 (or POD 250 and 280 in Group 5). The specimens were divided as follows: One part of the graft was fixed in 10% neutral-buffered formalin, embedded in paraffin and sections stained with hematoxylin and eosin (H&E) for histological examination. The second part of the graft was snap frozen in liquid nitrogen, and serially sectioned in cryostat. Immunostaining for CD4-, CD8-cells, B220+ B cells, CD11b+ macrophages and insulin was performed to identify the phenotype of the infiltrating cells.

In addition, sections of pancreases from Group 1 on POD 30 as well as Group 5 animals on POD 280 were stained for insulin.

Group 1 and 5 mice were also examined for evidence of graft versus host disease (GVHD). Therefore, H&E-tissue sections were generated and examined from the lungs, the skin, the small intestine and the tongue.

Anti-donor Interferon-gamma-Analysis

Enzyme-linked Immunosorbent Spot (ELISPOT) assays were performed on POD 30 to determine the frequency of antigen-specific T cells secreting interferon-gamma (IFN-γ) in response to Balb/c and NOD cells (48; 57). ELISPOT plates (Cellular Technology Ltd., Cleveland, OH) were prepared with 100 µl coating antibodies for IFN-γ (4 µg/ml, PharMingen, San Diego, CA) per well and incubated at 4 ºC overnight. The plates were thereafter blocked with PBS-1% bovine serum album (BSA, Sigma, St. Louis, MO) and washed three times with PBS. Spleens were harvested from individual Group 1, 2 and 7 mice as well as untreated littermates and 0.5×10^6 splenic mononuclear cells were added per well. After addition of an equal number of irradiated (3 Gy) T cell-depleted stimulator cells in HL-1 medium (BioWhittaker, Walkersville, MD), the plates were incubated at 37 ºC and 5% CO_2 for

24 hours. Cells were removed and wells were washed with PBS-0.025% Tween. Biotinylated rat anti-mouse detection antibodies for IFN-γ (1 to 5 µg/ml, PharMingen, San Diego, CA), diluted in PBS-Tween-1% BSA, were added to each well and the ELISPOT plate was incubated overnight at 4 ºC. Plates were washed and incubated for 90 minutes at room temperature with streptavidin-horseradish peroxidase (Dako Corporation, Carpinteria, CA), diluted in PBS-Tween-1% BSA. Spots were developed with 3-amino-9-ethylcarbazole (AEC, 10 mg/ml in N,N-dimethylformamide, Pierce, Rockford, IL) and then counted on a computer-assisted Immunospot Image Analyzer (Cellular Technologies, Ltd., Cleveland, OH).

Resident Peritoneal Macrophage Culture

Resident peritoneal cells (PEC) were cultured as previously described (58). PEC from chimeric (Group 8) or immunosuppressed (Group 9) mice were harvested one day following islet transplantation by three injections of 5 ml PBS. PEC were resuspended in RPMI-1640, 1 x 10^{-2} M morpholinopropane sulfonic acid, 5 x 10^{-5} M 2-mercaptoethanol (2-ME), BSA (2.5 mg/ml), transferrin (10 µg/ml) and insulin (1 µU/ml). Tissue culture reagents were obtained from Life Technologies (Gaithersburg, MD) or Sigma (St. Louis, MO). This medium was purposely employed to avoid the contribution of transforming growth factor–beta 1 (TGF-ß1) found in fetal bovine serum (FBS) and other serum supplement. All medium components contained less than 0.0015 ng endotoxin/ml. PEC were plated at 3 x 10^6/ml in 0.2 ml in replicate microtiter wells, incubated at 37 ºC in an atmosphere of 7% CO_2 for 48 hours and supernatants were collected. Experiments, comparing PEC from chimeric and immunosuppressed mice, were “head to head”, being performed on the same day with the same media and reagents.

Transforming growth factor-beta1-Determination

TGF-ß1 in PEC supernatants was measured using a commercially available kit (R&D Systems, Minneapolis, MN). Total TGF-β1 (active and latent) was measured; the sensitivity limit of the assay was 7 pg/ml.

Statistical Analysis

The time course and diabetes reversal rates of the immunosuppressed and chimeric mice were compared for significant differences by Kaplan-Meier analysis. Results of the IPGTT and the TGF-ß1 levels were expressed as mean +/- standard error of mean. Student's t-test was used to determine the significance of differences between control and treatment groups in anti-donor T cell frequencies in ELISPOT assays as well as the glucose areas under the curve in IPGTTs. P values <0.05 were considered statistically significant.

Results

Significantly reduced islet number required to achieve normoglycemia in chimeric NOD mice compared to immunosuppressed animals

All diabetic NOD mice made tolerant pretransplant with the mixed chimerism protocol named above achieved normoglycemia following transplantation of 200 (9/9), 300 (8/8) or 400 (8/8) Balb/c islets (Table 1; Figure 3b-d, 4b). Of the chimeric mice receiving 100 islets, 83% (5/6) became normoglycemic by POD 30 (Table 1; Figure 3a, 4a). In marked contrast, it was necessary to transplant 400 islets to immunosuppressed mice to restore normoglycemia in about the same proportion of recipients (6/8) (Table 1; Figure 3d). Transplanting fewer islets, 300 or 200 (Table 1; Figure 3b/c, 4d), into immunosuppressed mice further decreased the percentage of normoglycemic mice and significantly fewer animals achieved stable normoglycemia posttransplantation ($p<0.05$ for 300 islets, $p<0.001$ for 200 islets). None of the immunosuppressed mice receiving 100 islets became persistently normoglycemic (Table 1; Figure 3a, 4c, $p<0.05$ vs. chimeric animals).

Tight posttransplant glucose control in tolerant mice in contrast to animals treated similar to the 'Edmonton' protocol

Thirty days following islet transplantation, an IPGTT was performed on fasted chimeric and immunosuppressed mice that were normoglycemic by postoperative day 30. Chimeric mice that had received 100 to 400 islets maintained normoglycemia throughout the entire test (Figure 5). In contrast, islets transplanted into immunosuppressed recipients clearly exhibited less glucose control. This was particularly evident in the small percentage of immunosuppressed mice that achieved normoglycemia with 200 islets (16.7%, 2/12), which became hyperglycemic with mean glucose levels above 300 mg/dl by 10 minutes after glucose administration.

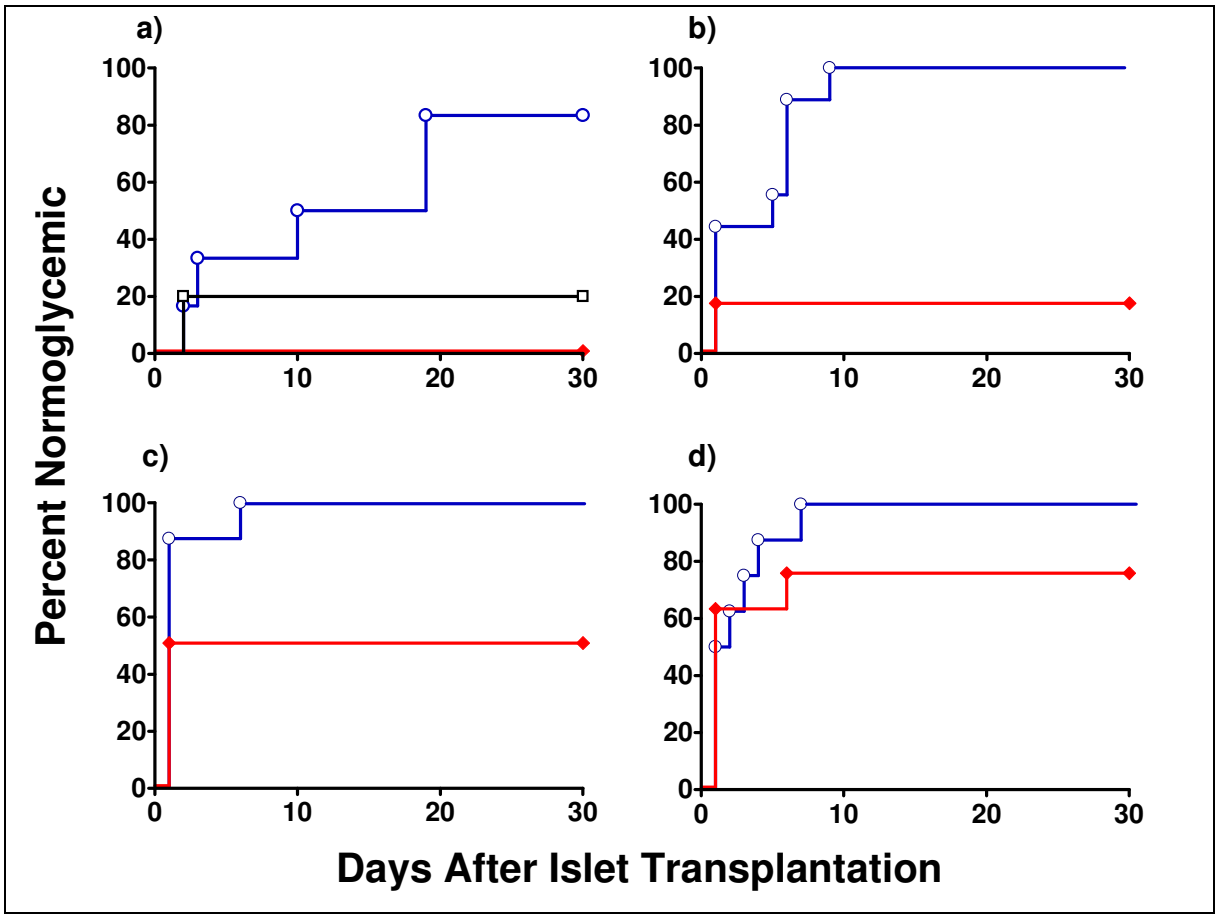

Figure 3. Percentage of persistent normoglycemia in chimeric (blue) versus immunosuppressed (red) NOD mice after transplantation of a) 100 ($p<0.05$), b) 200 ($p<0.001$), c) 300 ($p<0.05$) and d) 400 ($p=0.33$) allogeneic Balb/c islets below the kidney capsule. The addition of sirolimus (1 mg/kg/day) and low-dose tacrolimus (0.5 mg/kg/day) to NOD mice with established mixed chimerism reduced the percentage of normoglycemic animals from 83% (5/6) to 20% (1/5; $p=0.08$) with a 100 Balb/c islet graft (a, black line).

Graft morphology showed intact islets in all grafts, but is markedly different in chimeric and immunosuppressed mice

The analysis of islet grafts in chimeric recipients in Groups 1 and 4 on POD 30 and in Group 5 on POD 250 and 280 showed numerous, well-preserved and well-granulated islets with no evidence of cellular infiltration or scattered peri-islet infiltration of CD4+ T cells and macrophages (Figure 6 a, b; 7 a-d). Analyses of islet grafts in immunosuppressed Group 2 recipients showed a dense, peri-islet cellular infiltrate without disruption of the islet architecture (Figure 6 c and d).

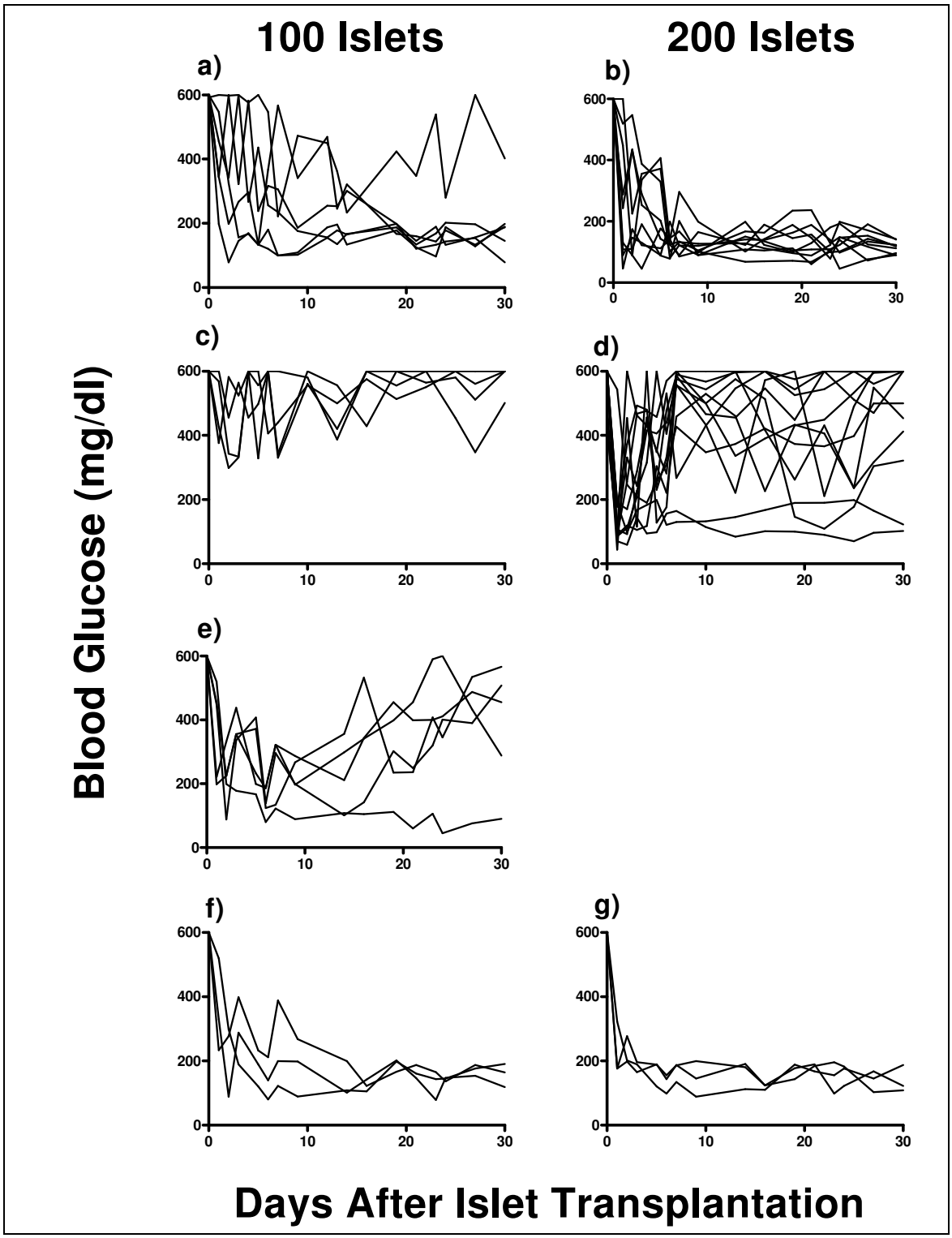

Figure 4. Non-fasting plasma glucose levels after transplantation of 100 (left panel) and 200 (right panel) Balb/c islets below the kidney capsule. The individual lines represent the plasma glucose levels of each animal. Recipient animals were chimeric (a,b), received 'Edmonton' like immunosuppression (c,d) and were given sirolimus (1 mg/kg/day) and tacrolimus (0.5 mg/kg/day) in addition to established mixed hematopoietic chimerism (e). After removal of the islet-bearing left kidney 250 days after transplantation, chimeric animals received a second islet transplant below the right kidney capsule (here POD 0) following the return to hyperglycemia (f, g).

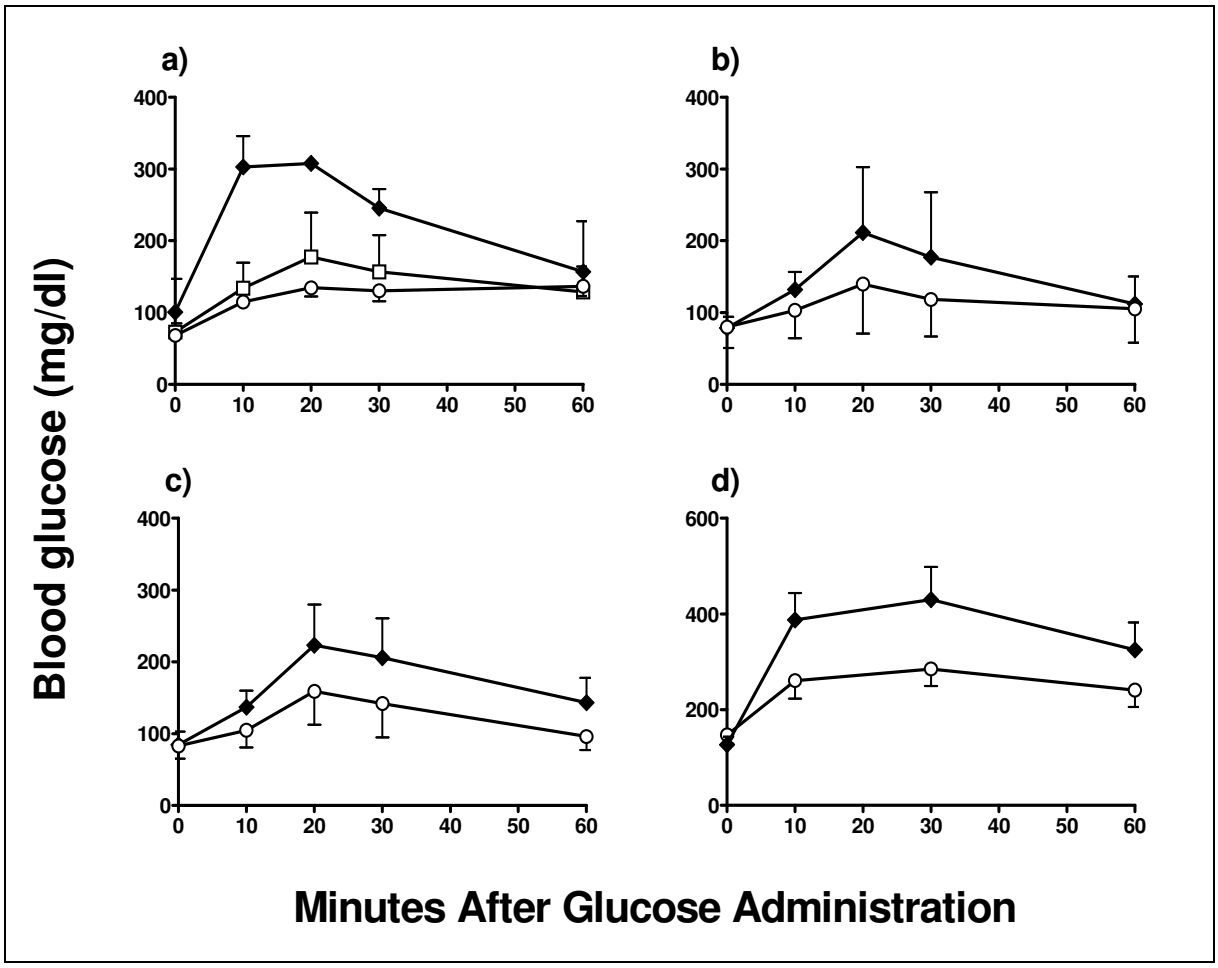

Figure 5. An IPGTT was performed 30 days after islet transplantation in persistent normoglycemic animals following the transplantation of a) 100 islets (n=3; open squares) as well as a) 200 (n=7), b) 300 (n=3) and c) 400 (n=5) islets in chimeric (open circles) NOD mice. IPGTTs in immunosuppressed, normoglycemic NOD mice following the transplantation of a) 200 (n=2; p=0.16 vs. chimeric), b) 300 (n=4; p=0.77) and c) 400 (n=5; p=0.35) islets are shown with closed diamonds. D) IPGTT in B6 mice with stable, low-numbered isografts before (open circles) and after (closed diamonds) 30 days of administration of sirolimus (1 mg/kg/day) and tacrolimus (0.5 mg/kg/day; p=0.12). Values are shown as averages with standard error of the mean.

The infiltrate was comprised predominantly of CD4+ T cells and, to a lesser extent, also of CD8+ T cells, macrophages and B cells (Figure 7 e-h).

Histological examination of islet grafts in non-chimeric, non-immunosuppressed Group 7 animals revealed only a few islet remnants with massive infiltration of mononuclear cells (Figure 6 e, f). Staining for insulin in pancreases of chimeric animals on POD 30 (Group 1) as well as POD 280 (Group 5) revealed no viable islet cells (data not shown).

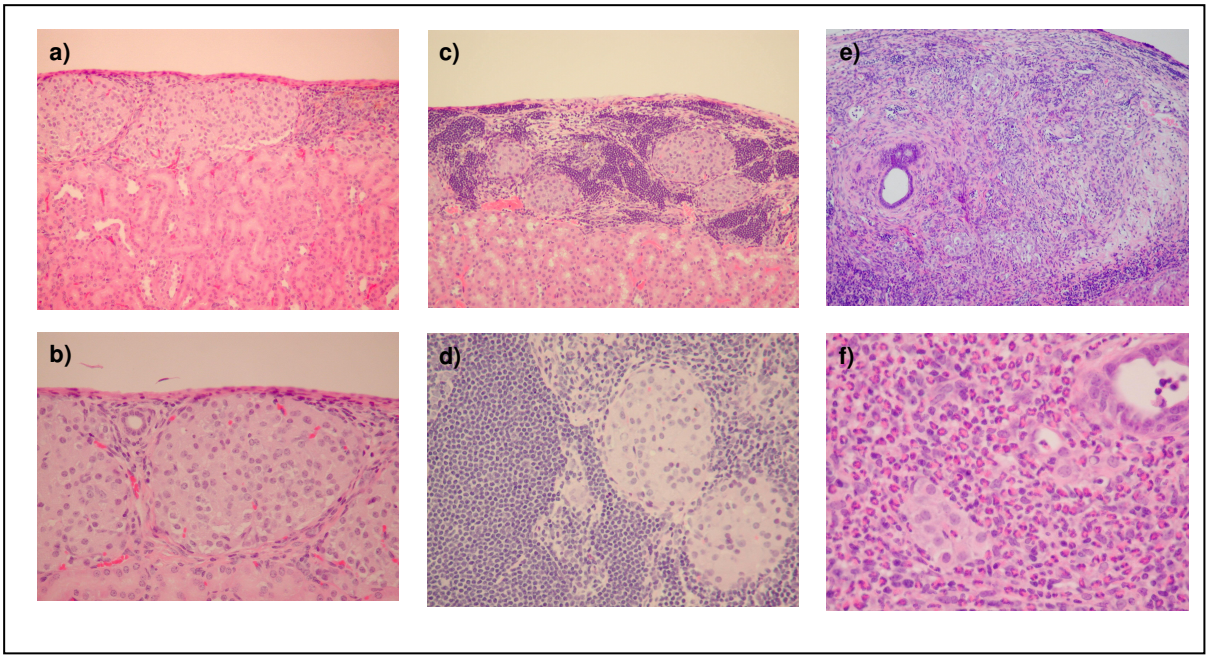

Figure 6. Histological analysis (H&E) of islet Balb/c grafts in chimeric (a, b), immunosuppressed (c, d) and untreated NOD mice (e, f). An immense peri-islet infiltrate in grafts of immunosuppressed animals was noticeable, whereas untreated mice rejected their transplants and only islet remnants could be seen on POD 30.

Tolerance induction and immunosuppression markedly reduce anti-donor T cell responses

Balb/c islet transplantation into untreated diabetic NOD recipients caused a marked rise in Balb/c antigen-specific splenic IFN-γ-positive lymphocytes as measured by a rise from 1.4 IFN-γ ELISA spots/5 x 10^5 cells pretransplant to 50.4 spots at POD 30 (Figure 8). In striking contrast, spleen cells from chimeric NOD mice only exhibited very low IFN-γ levels upon stimulation with Balb/c antigens (1.33 spots) at POD 30. In comparison to untreated mice, immunosuppression also caused a reduction of donor antigen-specific IFN-γ-secreting cells with normoglycemic recipients averaging 5.4 spots ($p=0.2$ vs. chimeric) and hyperglycemic animals 7.2 spots ($p=0.01$ vs. chimeric). There was no significant difference in the anti-donor T cell responses between immunosuppressed animals that achieved persistent normoglycemia and those who did not ($p=0.63$).

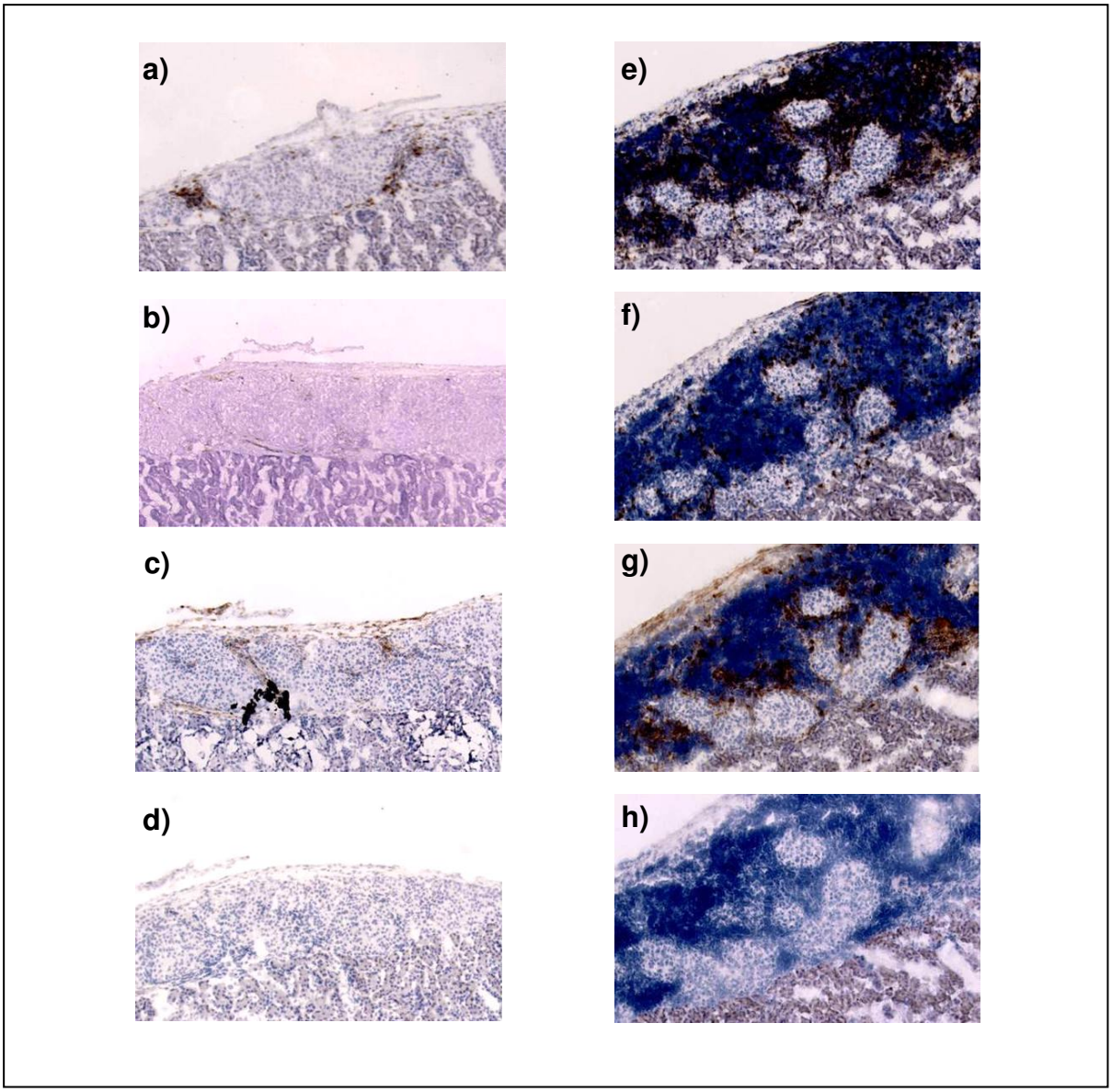

Figure 7. Paired photomicrographs of immunohistochemical labeling for CD4 (a, e), CD8 (b, f), CD11b (c, g) and B220 (d, h) in chimeric (left panel) and immunosuppressed (right panel) NOD mice 30 days after islet transplantation. Islet grafts in chimeric mice had no or a minimal infiltrate, whereas grafts of immunosuppressed animals demonstrated numerous CD4+ T as well as B cells and macrophages surrounding the islet grafts. Islets in both groups appeared well-preserved.

Immunosuppression slightly influences stable islet isografts, but has detrimental effects in the early posttransplant period of islet allografts

To test the diabetogenic effects of the immunosuppressive regimen used in this study, tacrolimus and SIR were administered to stable C57BL/6 islet isograft

recipients over 30 days in the same concentration as in Group 2 animals. Non-fasting blood glucose levels did not alter before and after this treatment (159.18 mg/dl ± 35.6 before versus 159.96 mg/dl ± 56.5 after immunosuppression). However, an intraperitoneal glucose tolerance test revealed that a 30 day course of immunosuppression showed a trend towards decreased islet function in these mice (Figure 5d; p=0.12).

In contrast to the lack of a strong inhibitory effect of immunosuppression on an established islet graft, this same treatment reduced the ability of 100 allogeneic islets (Group 4) to restore normoglycemia in NOD recipients with mixed hematopoietic chimerism from 83% (5/6) to 20% (1/5; p=0.08, Figure 3a, 4e).

Recurrence of hyperglycemia in long-term chimeric NOD mice was promptly reversed after transplantation of a second graft. Chimeric animals rejected third-party islet grafts.

The procedures, immunosuppressive regimens and conditioning therapy in this study were well tolerated. No signs of GVHD were evident by histological analyses of the lungs, skin, small intestine and tongue in chimeric NOD mice on POD 30 or 280 (data not shown).

In Group 5, posttransplant monitoring was extended to POD 250. All recipients of low-dose islet-allografts (100 or 200 islets) showed stable normoglycemia through POD 250 at which time they underwent graft nephrectomy. After return to hyperglycemia, these animals were retransplanted with 100 or 200 Balb/c islets to the right kidney. Again, all mice became normoglycemic and remained so until POD 280 (Figure 4f, g). Third-party C3H islets transplanted to chimeric NOD mice on POD 0 (Group 7) were rejected by day 22 (Table 1).

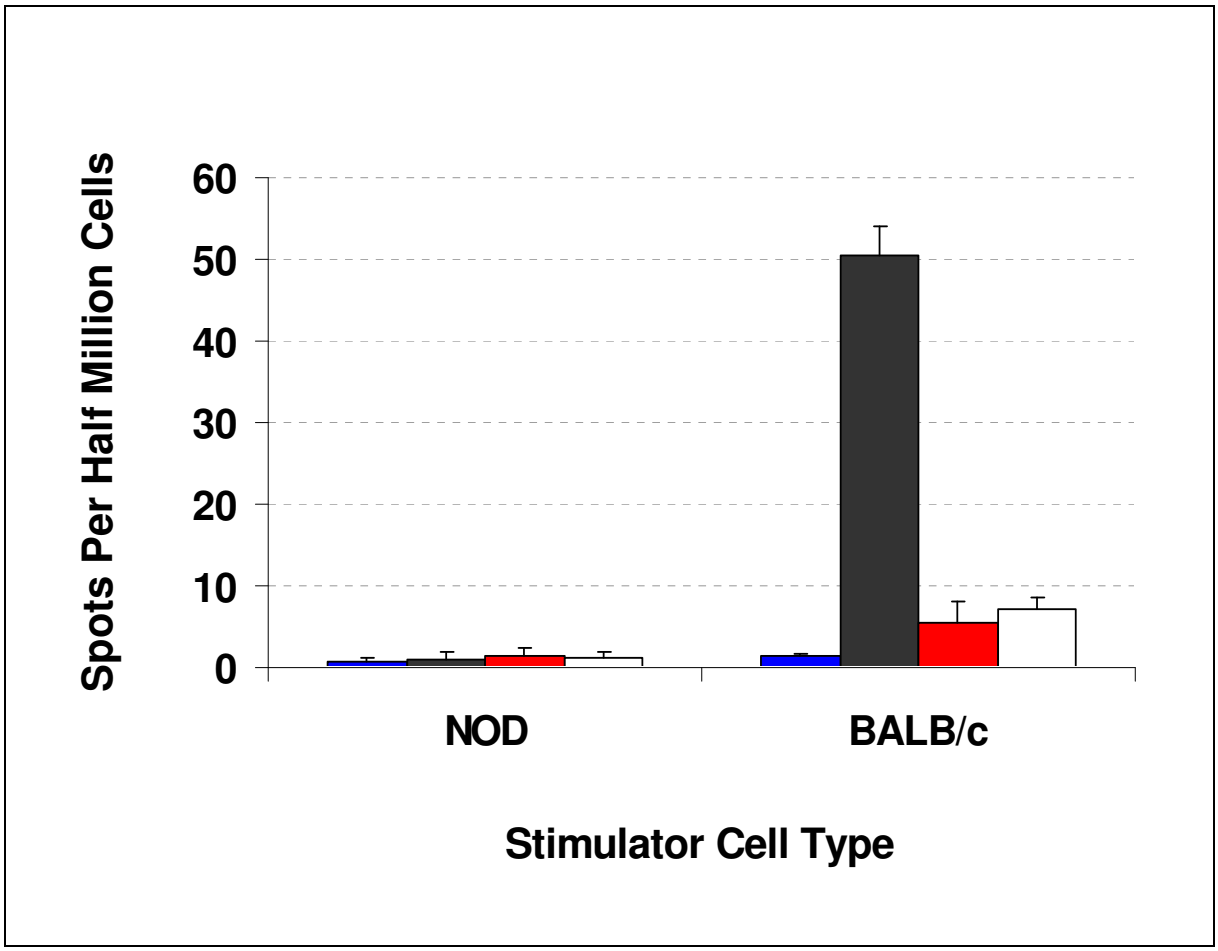

Figure 8. An ELISPOT assay for IFN-γ producing cells of six chimeric (blue bar), two untreated, antigen exposed (black bar) as well as five immunosuppressed normoglycemic (red bar) and five hyperglycemic (white bar) NOD mice in response to syngeneic NOD stimulator cells and donor-strain Balb/c antigens was performed 30 days after the transplantation of islet cells. Values are shown as averages with standard error of the mean.

Slightly elevated TGF-ß1 levels in chimeric animals compared to immunosuppressed NOD mice

To examine the role of the immunoregulatory cytokine TGF-ß1 in the early posttransplant period, PEC of chimeric (Group 8) as well as immunosuppressed (Group 9) animals were recovered to measure TGF-ß1 production of peritoneal macrophages one day after islet transplantation. Figure 9 demonstrates that chimeric mice exhibited elevated TGF-ß1 levels in comparison to immunosuppressed islet recipients. However, the difference was not significant ($p=0.49$) and TGF-ß1 production did not correlate with chimerism levels (data not shown).

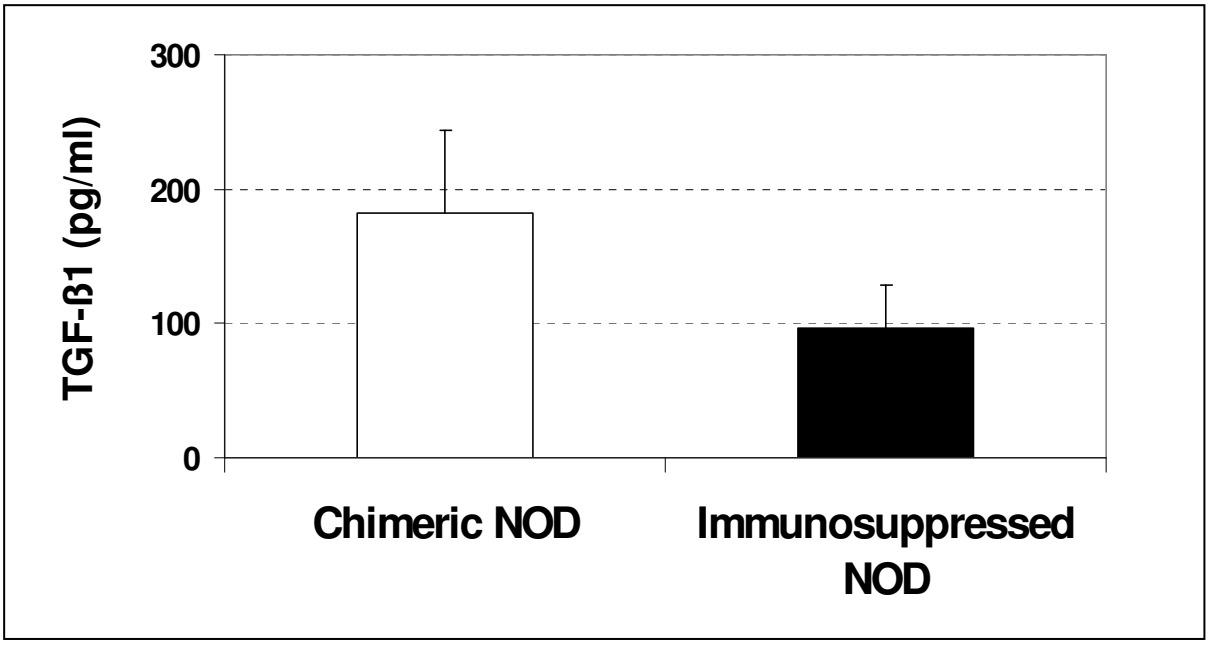

Figure 9. TGF-ß1 production in NOD mice with chimerism levels >20% (white bar) and immunosuppressed animals (black bar) were determined in resident peritoneal macrophages one day after islet transplantation (p=0.49). Values are shown as averages with standard error of the mean.

Discussion

Transplantation of islets of Langerhans can safely restore endocrine function and has the potential of preventing and even reversing diabetic long-term complications (59) as seen in pancreas transplant recipients (60). However, due to the high islet number required in the 'Edmonton protocol', only 0.5% of all diabetic patients could be treated (61).

The results of this study demonstrate that in order to achieve stable diabetes reversal with a probability of about 80%, fourfold less islets are required after islet allotransplantation in diabetic NOD mice made tolerant prior to transplantation. The islet mass that was found to be sufficient to restore normoglycemia in chimeric mice was also low in comparison to previous reports of experimental islet allotransplantation in NOD mice with 500 to 700 islets (62-68). With the exception of the specific antibody used for induction immunosuppression, the immunosuppressive regimen applied in this study has been the standard protocol in clinical islet transplantation since the report of the Edmonton group in 2000 (16). The findings of this study suggest that this currently used immunosuppression protocol greatly compromises the ability of marginal mass human islet transplants to restore normoglycemia in type 1 diabetics. An improved understanding of the mechanisms causing the strikingly different diabetes reversal rates in tolerant, non-immunosuppressed versus immunosuppressed islet allograft recipients will be necessary to achieve the clinically-needed improvement in the marginal mass islet transplant success rate. In the experiments presented here, the failure of immunosuppressed animals to become normoglycemic after low-dose islet transplantation seems to be primarily due to inhibitory effects of tacrolimus and SRL on islet engraftment and not due to inhibition of islet function or sub-optimal immune

response suppression. This distinction can be critical to the design of future immunotherapeutic regimens.

The detrimental effects of combined administration of glucocorticoids and calcineurin inhibitors (CNI) such as cyclosporine-A (CsA) and tacrolimus on insulin secretion and sensitivity in islet transplant recipients are well documented (69). Shapiro et al. replaced glucocorticoids with SRL and a lowered tacrolimus dose. The enhanced islet transplantation results with this new protocol were ascribed to its reduced diabetogenicity in addition to an increased islet mass (16). However, recent studies reported an unexpected high frequency of new-onset diabetes after kidney transplantation even with glucocorticoid-free protocols (70). Also, the rate of diabetes development was greater when SRL was added to tacrolimus (70). SRL has inhibitory effects on insulin secretion in isolated islets (71-74) and induces insulin resistance in vivo (75-77). Furthermore, the combination of low-dose tacrolimus with SRL has been shown to cause diabetes in healthy rats (78). However, Shapiro and co-workers have demonstrated that the combination of low doses of sirolimus and tacrolimus in NOD mice had a strong synergistic effect in preventing diabetes recurrence following syngeneic islet transplantation (79).

To test the potential diabetogenic effects of the glucocorticoid-free combination of tacrolimus and SRL on islets independently of any islet immune response, SRL and tacrolimus were administered to normoglycemic animals that were bearing a stable minimal mass of syngeneic islets. The IPGTT after 30 days of drug administration revealed a trend towards decreased glucose tolerance. However, the non-fasting glucose levels did not change, suggesting no major inhibitory effect on stable islet grafts although the mice only received a transplant of 75 or 100 islet cells.

Conversely, the administration of the same regimen starting on POD -2 in relation to islet transplantation to tolerant NOD mice (Group 5) lowered the diabetes reversal

rate of 100-islets allografts from 83% in Group 1 to 20%. Thus, these results demonstrate the striking inhibitory effect on islet engraftment during the early posttransplant period.

After the isolation process, pancreatic islets are completely avascular and it has been shown that vascularization begins two to four days after islet transplantation and 10 to 14 days are needed to establish a new microvascular network (80; 81). The graft becomes revascularized from intra-islet as well as recipient-derived endothelial cells (82-84). SRL is known to inhibit the proliferation and differentiation of endothelial cells (85-87) and, with relevance to islet transplantation, it has been demonstrated to inhibit the outgrowth of endothelial cells from freshly isolated human islets along with the formation of capillary-like structures in vitro and in vivo (88; 89). Furthermore, SRL showed a dose-dependent adverse impact on engraftment of syngeneic and allogeneic islets in murine models (56; 74), plus caused a dose-dependent incidence of primary nonfunction after hamster-to-mouse islet xenotransplantation (90).

Antiangiogenic properties of CNI have been shown in vitro and in vivo as well (88; 91; 92). In murine models, CsA inhibited vascular ingrowth into transplanted islets (93) and decreased the graft insulin content (94). In a canine intrasplenic islet autograft model, four of six CsA-treated recipients showed graft failure by POD 6, whereas normoglycemia persisted in all eight non-immunosuppressed autografted control-animals until the end of the study (35). However, when CsA was started on POD 10, all islet recipients remained normoglycemic for more than 30 days (95).

Conversely, both CNI and SRL inhibit islet engraftment, and the profound impairment of early islet allograft function presented here suggests that tacrolimus and SRL synergize in inhibiting islet engraftment. It is also conceivable that islets are more sensitive to direct toxic effects mediated by CNI (96-101) and SRL (71; 72; 74) until full vascular incorporation has occurred (95). These effects might be even more

important in the setting of intraportal islet transplantation, since peak immunosuppressant levels as well as the area under the curve in portal blood relative to systemic levels for orally administered drugs have been shown to be dramatically elevated (102).

Immunologic graft loss as a reason for hyperglycemia in a subgroup of immunosuppressed animals was excluded through splenic lymphocytes IFN-γ responses and histology of all grafts. As a Th1 cytokine, IFN-γ appears to play a critical role in islet destruction following transplantation as well as in autoimmune diabetes (103-108). Specifically, this is the principal cytokine that activates macrophages to produce nitric oxide (NO) and other islet-noxious products that are upregulated in type 1 diabetic mice (109; 110) and rats (111). In line with these observations, there was a marked increase in the frequency of Balb/c donor antigen-specific IFN-γ-positive splenic lymphocytes in untreated NOD islet recipients. In contrast, in NOD recipients of Balb/c bone marrow that exhibited >40% donor chimerism, the frequency of IFN-γ-positive splenic lymphocytes was reduced to background levels. Thus, strong donor-specific tolerance was achieved in these chimeric mice as previously observed in chimeric NOD mice prepared using irradiation and more intensive immunosuppression (112). The frequency of IFN-γ-positive lymphocytes was also markedly reduced in NOD islet recipients treated with contemporary immunosuppression. Moreover, there was no significant difference in the number of IFN-γ-positive lymphocytes between immunosuppressed animals that were normoglycemic and those that never achieved persistent normoglycemia. Therefore, although the mechanisms responsible for the reduction in IFN-γ-positive cells in chimeric and immunosuppressed mice may have been different, both treatments effectively reduced this response.

The foregoing interpretation is supported by histological analyses of the islet grafts. Intact islets could be found in all mice regardless of whether they achieved normoglycemia or not. In contrast, histological examinations of islet grafts in non-chimeric, non-immunosuppressed Group 7 animals revealed only a few islet remnants with massive infiltration of mononuclear cells. However, islet grafts in chimeric mice showed minimal or no leukocyte infiltration, whereas immunostaining of grafts in the immunosuppressed group demonstrated an extensive infiltration of predominantly CD4+ and CD8+ lymphocytes surrounding the islets. Peri-islet infiltrations have been demonstrated in functioning islet (63; 79; 113) as well as in pancreas (114) grafts before. Infiltrates can also be observed in NOD mice and Gazda et al. discriminated between benign and malignant autoimmunity (115). The group reported that no destruction of beta cells or diabetes development occurs during the benign phase, despite profound insulitis. Green et al. showed that mainly Th2 cytokine expression was present in these benign infiltrations, thus demonstrating the non-aggressive nature (116). Others revealed immunoregulatory cells in these infiltrates (117). Therefore, these findings suggest that benign peri-islet infiltrates have no major adverse influence on functioning islet cells; however, the exact impact on islet cell function still needs to be determined.

Rejection of third-party cells in Group 7 mice showed immunocompetence of chimeric animals. Normoglycemia for 250 days after islet transplantation in Group 5 animals demonstrated the robust donor-specific tolerance and reversed autoimmunity. Furthermore, following the return to hyperglycemia after nephrectomy of the graft-bearing left kidney, transplantation of 100 or 200 Balb/c islets resulted in normoglycemia in all mice. In turn, these findings demonstrate that the superior outcomes in tolerant, non-immunosuppressed Group 1 mice compared with Group 2

recipients was not due to conditioning therapy nor anti-CD154 monoclonal antibody (mAb) exposure. Kiener (118) and Dechanet (119) described the release of islet-noxious nonspecific inflammatory mediators upon stimulation of human monocytes and endothelial cells via the CD40 pathway. Others demonstrated the beneficial effect of anti-CD154 treatment on islet survival in murine (120) and rhesus monkey models (121). Quesenberry et al. reported that hamster anti-CD154 mAb levels were detectable in murine blood for up to 15 weeks, but could not detect any levels 36 weeks after initial mAb course (122). Although the retransplantations of low-dose islet allografts in Group 5 animals were performed about 260 days, i.e. about 37 weeks, after the last administration of anti-CD154, the diabetes reversal rates were comparable to those observed in Group 1 animals. Thus, an influence of conditioning therapy and anti-CD154 treatment on diabetes reversal rates was excluded.

In addition, the return to hyperglycemia after nephrectomy of the graft-bearing kidney on POD 30, 250 or 280 excluded insulin production outside the graft and pancreatic beta cell regeneration (123). These results were further confirmed by immunohistological examinations of pancreases with no positive staining for insulin (data not shown).

Initial experiments found the production of TGF-ß1 by resident PEC one day after islet transplantation to be elevated in NOD islet recipients, especially when high chimerism levels were exhibited. Since elevated TGF-ß1 production was found in PEC of chimeric NOD recipients away from the kidney capsule site of islet implantation, the results suggested that the induction of chimerism induced a systemic increase in leukocyte TGF-ß1. This cytokine is now recognized to play a key immunoprotective role in NOD mice (124; 125) and in islet transplantation (113;

126). Specifically, it has been demonstrated that the release of TGF-ß in islet cells can prevent autoimmune diabetes via expansion of regulatory T cells in transgenic NOD mice (125). Co-transplantation of TGF-ß-producing Sertoli cells with islet cells resulted in a significant lower isograft rejection rate in NOD mice (113). Furthermore, macrophage islet–protective TGF-ß1 production has been shown to be inversely related to macrophage production of islet-toxic NO (58). Thus, a systemic elevation of TGF-ß1 could have a beneficial impact on islet graft survival. However, further experiments could only demonstrate a non-significant increase of TGF-ß1 production in chimeric NOD mice and there was no correlation between percentage of chimerism and TGF-ß levels (data not shown). It is known that TGF-ß1 and NO production vary between mice strains (58), with NOD mice exhibiting decreased TGF-ß1/NO ratios (111). Balb/c macrophages have been shown to be high producers of TGF-ß1 (58), thus it was conceivable that, after transplantation of Balb/c bone marrow into NOD mice, islet-protective TGF-ß1 levels could be elevated in the host. However, Shapiro et al. have shown that the combination therapy of sirolimus and tacrolimus in NOD mice with islet grafts resulted in upregulated TGF-ß1 expression as well (79), making an important role of this cytokine in the experimental setting presented here unlikely. Future studies need to determine whether mixed chimerism induces the release of other factors that would promote islet engraftment and function.

This study demonstrated that an extraordinary low mass of allogeneic islets is sufficient to restore normoglycemia in recipients with autoimmune diabetes if islet engraftment is allowed to occur in the absence of both adaptive immunity and immunosuppressive drug toxicity. These findings corroborate and extend the report by Kumagai et al., who demonstrated improved allograft function if islets were

allowed to revascularize in the absence of alloimmunity and CsA (36). The group prevascularized isolated islets under the autologues kidney before subsequent transplantation as a composite islet-kidney allograft into allogeneic, immunosuppressed dogs. Considering the current high demand of islet cells to achieve normoglycemia in humans, a reduction of the number of islets would make the available donor pancreases accessible to more diabetic patients. Furthermore, if an as high reduction of the islet number as seen in these experiments could be achieved in a clinical setting, in the future, living-donor transplantation (24) might be a possible treatment for diabetic patients on a regular basis.

In summary, combined administration of tacrolimus and SRL has detrimental effects on islet engraftment. Avoiding these drugs in the early posttransplant period by pretransplant induction of donor-specific tolerance and restoration of self-tolerance facilitates diabetes reversal with a very low islet mass. It is conceivable that other tolerance and immunotherapeutic strategies that control adaptive immunity without interfering with islet engraftment will be equally effective. First successful tolerance induction protocols, using successive bone marrow and kidney transplantation for the treatment of myeloma patients with chronic kidney failure, are currently being investigated in clinical trials (127). Extended administration of depleting T cell antibodies facilitating delayed initiation of CNI or SRL (22) or substitution of non-angiocidal immunotherapeutics for CNI and SRL (121) are likely to improve the success rate of marginal mass human islet allografts.

References

1. Daneman D: Type 1 diabetes. *Lancet* 367:847-858, 2006

2. Atkinson MA, Maclaren NK: The pathogenesis of insulin-dependent diabetes mellitus. *N Engl J Med* 331:1428-1436, 1994

3. Chervonsky AV, Wang Y, Wong FS, Visintin I, Flavell RA, Janeway CA, Jr., Matis LA: The role of Fas in autoimmune diabetes. *Cell* 89:17-24, 1997

4. Green EA, Flavell RA: The initiation of autoimmune diabetes. *Curr Opin Immunol* 11:663-669, 1999

5. Horwitz MS, Bradley LM, Harbertson J, Krahl T, Lee J, Sarvetnick N: Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. *Nat Med* 4:781-785, 1998

6. Nathan DM: Long-term complications of diabetes mellitus. *N Engl J Med* 328:1676-1685, 1993

7. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. *N Engl J Med* 329:977-986, 1993

8. Nordfeldt S, Ludvigsson J: Fear and other disturbances of severe hypoglycaemia in children and adolescents with type 1 diabetes mellitus. *J Pediatr Endocrinol Metab* 18:83-91, 2005

9. Kelly WD, Lillehei RC, Merkel FK, Idezuki Y, Goetz FC: Allotransplantation of the pancreas and duodenum along with the kidney in diabetic nephropathy. *Surgery* 61:827-837, 1967

10. Larsen JL: Pancreas transplantation: indications and consequences. *Endocr Rev* 25:919-946, 2004

11. Sutherland DE, Gruessner RW, Dunn DL, Matas AJ, Humar A, Kandaswamy R, Mauer SM, Kennedy WR, Goetz FC, Robertson RP, Gruessner AC, Najarian JS:

Lessons learned from more than 1,000 pancreas transplants at a single institution. *Ann Surg* 233:463-501, 2001

12. Merani S, Shapiro AM: Current status of pancreatic islet transplantation. *Clin Sci (Lond)* 110:611-625, 2006

13. Lakey JR, Warnock GL, Brierton M, Ao Z, Hering BJ, London NJ, Ricordi C, Corbin F, Rajotte RV: Development of an automated computer-controlled islet isolation system. *Cell Transplant* 6:47-57, 1997

14. Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW: Automated method for isolation of human pancreatic islets. *Diabetes* 37:413-420, 1988

15. Ballinger WF, Lacy PE: Transplantation of intact pancreatic islets in rats. *Surgery* 72:175-186, 1972

16. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 343:230-238, 2000

17. Markmann JF, Deng S, Huang X, Desai NM, Velidedeoglu EH, Lui C, Frank A, Markmann E, Palanjian M, Brayman K, Wolf B, Bell E, Vitamaniuk M, Doliba N, Matschinsky F, Barker CF, Naji A: Insulin independence following isolated islet transplantation and single islet infusions. *Ann Surg* 237:741-749; discussion 749-750, 2003

18. Goss JA, Schock AP, Brunicardi FC, Goodpastor SE, Garber AJ, Soltes G, Barth M, Froud T, Alejandro R, Ricordi C: Achievement of insulin independence in three consecutive type-1 diabetic patients via pancreatic islet transplantation using islets isolated at a remote islet isolation center. *Transplantation* 74:1761-1766, 2002

19. Froud T, Ricordi C, Baidal DA, Hafiz MM, Ponte G, Cure P, Pileggi A, Poggioli R, Ichii H, Khan A, Ferreira JV, Pugliese A, Esquenazi VV, Kenyon NS, Alejandro R:

Islet transplantation in type 1 diabetes mellitus using cultured islets and steroid-free immunosuppression: Miami experience. *Am J Transplant* 5:2037-2046, 2005

20. Kaufman DB, Baker MS, Chen X, Leventhal JR, Stuart FP: Sequential kidney/islet transplantation using prednisone-free immunosuppression. *Am J Transplant* 2:674-677, 2002

21. Lehmann R, Weber M, Berthold P, Zullig R, Pfammatter T, Moritz W, Madler K, Donath M, Ambuhl P, Demartines N, Clavien And PA, Andreia Spinas G: Successful simultaneous islet-kidney transplantation using a steroid-free immunosuppression: two-year follow-up. *Am J Transplant* 4:1117-1123, 2004

22. Hering BJ, Kandaswamy R, Harmon JV, Ansite JD, Clemmings SM, Sakai T, Paraskevas S, Eckman PM, Sageshima J, Nakano M, Sawada T, Matsumoto I, Zhang HJ, Sutherland DE, Bluestone JA: Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. *Am J Transplant* 4:390-401, 2004

23. Hering BJ, Kandaswamy R, Ansite JD, Eckman PM, Nakano M, Sawada T, Matsumoto I, Ihm SH, Zhang HJ, Parkey J, Hunter DW, Sutherland DE: Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. *Jama* 293:830-835, 2005

24. Matsumoto S, Okitsu T, Iwanaga Y, Noguchi H, Nagata H, Yonekawa Y, Yamada Y, Fukuda K, Tsukiyama K, Suzuki H, Kawasaki Y, Shimodaira M, Matsuoka K, Shibata T, Kasai Y, Maekawa T, Shapiro J, Tanaka K: Insulin independence after living-donor distal pancreatectomy and islet allotransplantation. *Lancet* 365:1642-1644, 2005

25. Dunn DL: Problems related to immunosuppression. Infection and malignancy occurring after solid organ transplantation. *Crit Care Clin* 6:955-977, 1990

26. Teuscher AU, Kendall DM, Smets YF, Leone JP, Sutherland DE, Robertson RP: Successful islet autotransplantation in humans: functional insulin secretory reserve as an estimate of surviving islet cell mass. *Diabetes* 47:324-330, 1998

27. Pyzdrowski KL, Kendall DM, Halter JB, Nakhleh RE, Sutherland DE, Robertson RP: Preserved insulin secretion and insulin independence in recipients of islet autografts. *N Engl J Med* 327:220-226, 1992

28. Farney AC, Najarian JS, Nakhleh RE, Lloveras G, Field MJ, Gores PF, Sutherland DE: Autotransplantation of dispersed pancreatic islet tissue combined with total or near-total pancreatectomy for treatment of chronic pancreatitis. *Surgery* 110:427-437; discussion 437-429, 1991

29. Clayton HA, Davies JE, Pollard CA, White SA, Musto PP, Dennison AR: Pancreatectomy with islet autotransplantation for the treatment of severe chronic pancreatitis: the first 40 patients at the leicester general hospital. *Transplantation* 76:92-98, 2003

30. Alsaif F, Molinari M, Al-Masloom A, Lakey JR, Kin T, Shapiro AM: Pancreatic islet autotransplantation with completion pancreatectomy in the management of uncontrolled pancreatic fistula after whipple resection for ampullary adenocarcinoma. *Pancreas* 32:430-431, 2006

31. Stegall MD, Loberman Z, Ostrowska A, Coulombe M, Gill RG: Autoimmune destruction of islet grafts in the NOD mouse is resistant to 15-deoxyspergualin but sensitive to anti-CD4 antibody. *J Surg Res* 64:156-160, 1996

32. Terada M, Salzler M, Lennartz K, Mullen Y: The effect of H-2 compatibility on pancreatic beta cell survival in the nonobese diabetic mouse. *Transplantation* 45:622-627, 1988

33. Stegall MD, Lafferty KJ, Kam I, Gill RG: Evidence of recurrent autoimmunity in human allogeneic islet transplantation. *Transplantation* 61:1272-1274, 1996

34. Kaufman DB, Platt JL, Rabe FL, Dunn DL, Bach FH, Sutherland DE: Differential roles of Mac-1+ cells, and CD4+ and CD8+ T lymphocytes in primary nonfunction and classic rejection of islet allografts. *J Exp Med* 172:291-302, 1990

35. Merrell RC, Mahoney ME, Basadonna G, Cobb LF, Maeda M: Failure of canine islet allografts and autografts with cyclosporine. *Surgery* 98:324-329, 1985

36. Kumagai N, LaMattina JC, Kamano C, Vagefi PA, Barth RN, O'Neil JJ, Yamamoto S, Moran SG, Utsugi R, Sachs DH, Yamada K: Vascularized islet cell transplantation in miniature Swine: islet-kidney allografts correct the diabetic hyperglycemia induced by total pancreatectomy. *Diabetes* 51:3220-3228, 2002

37. Owen R: Immunogenetic consequences of vascular anastomoses between bovine twins. *Science* 102, 1945

38. Brent L: The discovery of immunologic tolerance. *Hum Immunol* 52:75-81, 1997

39. Billingham RE, Brent L, Medawar PB: Actively acquired tolerance of foreign cells. *Nature* 172:603-606, 1953

40. Ildstad ST, Wren SM, Bluestone JA, Barbieri SA, Sachs DH: Characterization of mixed allogeneic chimeras. Immunocompetence, in vitro reactivity, and genetic specificity of tolerance. *J Exp Med* 162:231-244, 1985

41. Wekerle T, Sykes M: Mixed chimerism as an approach for the induction of transplantation tolerance. *Transplantation* 68:459-467, 1999

42. Sachs DH: Mixed chimerism as an approach to transplantation tolerance. *Clin Immunol* 95:S63-68, 2000

43. Sykes M: Mixed chimerism and transplant tolerance. *Immunity* 14:417-424, 2001

44. Guo Z, Wu T, Sozen H, Pan Y, Heuss N, Kalscheuer H, Sutherland DE, Blazar BR, Hering BJ: A substantial level of donor hematopoietic chimerism is required to protect donor-specific islet grafts in diabetic NOD mice. *Transplantation* 75:909-915, 2003

45. Mathieu C, Casteels K, Bouillon R, Waer M: Protection against autoimmune diabetes in mixed bone marrow chimeras: mechanisms involved. *J Immunol* 158:1453-1457, 1997

46. Li H, Colson YL, Ildstad ST: Mixed allogeneic chimerism achieved by lethal and nonlethal conditioning approaches induces donor-specific tolerance to simultaneous islet allografts. *Transplantation* 60:523-529, 1995

47. Chakrabarti S, Handa SK, Bryon RJ, Griffiths MJ, Milligan DW: Will mixed chimerism cure autoimmune diseases after a nonmyeloablative stem cell transplant? *Transplantation* 72:340-342, 2001

48. Wu T, Sozen H, Luo B, Heuss N, Kalscheuer H, Lan P, Sutherland DE, Hering BJ, Guo Z: Rapamycin and T cell costimulatory blockade as post-transplant treatment promote fully MHC-mismatched allogeneic bone marrow engraftment under irradiation-free conditioning therapy. *Bone Marrow Transplant* 29:949-956, 2002

49. Durham MM, Bingaman AW, Adams AB, Ha J, Waitze SY, Pearson TC, Larsen CP: Cutting edge: administration of anti-CD40 ligand and donor bone marrow leads to hemopoietic chimerism and donor-specific tolerance without cytoreductive conditioning. *J Immunol* 165:1-4, 2000

50. Seung E, Iwakoshi N, Woda BA, Markees TG, Mordes JP, Rossini AA, Greiner DL: Allogeneic hematopoietic chimerism in mice treated with sublethal myeloablation and anti-CD154 antibody: absence of graft-versus-host disease, induction of skin allograft tolerance, and prevention of recurrent autoimmunity in islet-allografted NOD/Lt mice. *Blood* 95:2175-2182, 2000

51. Wekerle T, Kurtz J, Ito H, Ronquillo JV, Dong V, Zhao G, Shaffer J, Sayegh MH, Sykes M: Allogeneic bone marrow transplantation with co-stimulatory blockade

induces macrochimerism and tolerance without cytoreductive host treatment. *Nat Med* 6:464-469, 2000

52. Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y: Breeding of a non-obese, diabetic strain of mice. *Jikken Dobutsu* 29:1-13, 1980

53. Anderson MS, Bluestone JA: The NOD mouse: a model of immune dysregulation. *Annu Rev Immunol* 23:447-485, 2005

54. Gotoh M, Maki T, Kiyoizumi T, Satomi S, Monaco AP: An improved method for isolation of mouse pancreatic islets. *Transplantation* 40:437-438, 1985

55. Guo Z, Wu T, Kirchhof N, Mital D, Williams JW, Azuma M, Sutherland DE, Hering BJ: Immunotherapy with nondepleting anti-CD4 monoclonal antibodies but not CD28 antagonists protects islet graft in spontaneously diabetic nod mice from autoimmune destruction and allogeneic and xenogeneic graft rejection. *Transplantation* 71:1656-1665, 2001

56. Fabian MC, Lakey JR, Rajotte RV, Kneteman NM: The efficacy and toxicity of rapamycin in murine islet transplantation. In vitro and in vivo studies. *Transplantation* 56:1137-1142, 1993

57. Gebauer BS, Hricik DE, Atallah A, Bryan K, Riley J, Tary-Lehmann M, Greenspan NS, Dejelo C, Boehm BO, Hering BJ, Heeger PS: Evolution of the enzyme-linked immunosorbent spot assay for post-transplant alloreactivity as a potentially useful immune monitoring tool. *Am J Transplant* 2:857-866, 2002

58. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM: M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol* 164:6166-6173, 2000

59. Shapiro AM: Islet transplants and impact on secondary diabetic complications: does C-Peptide protect the kidney? *J Am Soc Nephrol* 14:2214-2216, 2003

60. Fioretto P, Steffes MW, Sutherland DE, Goetz FC, Mauer M: Reversal of lesions of diabetic nephropathy after pancreas transplantation. *N Engl J Med* 339:69-75, 1998

61. Shapiro AM, Ryan EA, Lakey JR: Diabetes. Islet cell transplantation. *Lancet* 358 Suppl:S21, 2001

62. Molano RD, Berney T, Pileggi A, Ricordi C, Burkly L, Rothstein D, Basadonna G, Inverardi L: Prolonged survival of allogeneic islet grafts in NOD mice treated with a combination of anti-CD45RB and anti-CD154 antibodies. *Transplant Proc* 33:248-249, 2001

63. Molano RD, Pileggi A, Berney T, Poggioli R, Zahr E, Oliver R, Malek TR, Ricordi C, Inverardi L: Long-term islet allograft survival in nonobese diabetic mice treated with tacrolimus, rapamycin, and anti-interleukin-2 antibody. *Transplantation* 75:1812-1819, 2003

64. Okitsu T, Bartlett ST, Hadley GA, Drachenberg CB, Farney AC: Recurrent autoimmunity accelerates destruction of minor and major histoincompatible islet grafts in nonobese diabetic (NOD) mice. *Am J Transplant* 1:138-145, 2001

65. Fu F, Hu S, Deleo J, Li S, Hopf C, Hoover J, Wang S, Brinkmann V, Lake P, Shi VC: Long-term islet graft survival in streptozotocin- and autoimmune-induced diabetes models by immunosuppressive and potential insulinotropic agent FTY720. *Transplantation* 73:1425-1430, 2002

66. Berney T, Pileggi A, Molano RD, Poggioli R, Zahr E, Ricordi C, Inverardi L: The effect of simultaneous CD154 and LFA-1 blockade on the survival of allogeneic islet grafts in nonobese diabetic mice. *Transplantation* 76:1669-1674, 2003

67. Makhlouf L, Duvivier-Kali VF, Bonner-Weir S, Dieperink H, Weir GC, Sayegh MH: Importance of hyperglycemia on the primary function of allogeneic islet transplants. *Transplantation* 76:657-664, 2003

68. Shi Q, Wang D, Hadley GA, Bingaman AW, Bartlett ST, Farber DL: Long-term islet graft survival in NOD mice by abrogation of recurrent autoimmunity. *Diabetes* 53:2338-2345, 2004

69. Shapiro AM, Geng Hao E, Lakey JR, Finegood DT, Rajotte RV, Kneteman NM: Defining optimal immunosuppression for islet transplantation based on reduced diabetogenicity in canine islet autografts. *Transplantation* 74:1522-1528, 2002

70. Sulanc E, Lane JT, Puumala SE, Groggel GC, Wrenshall LE, Stevens RB: New-onset diabetes after kidney transplantation: an application of 2003 International Guidelines. *Transplantation* 80:945-952, 2005

71. Paty BW, Harmon JS, Marsh CL, Robertson RP: Inhibitory effects of immunosuppressive drugs on insulin secretion from HIT-T15 cells and Wistar rat islets. *Transplantation* 73:353-357, 2002

72. Bell E, Cao X, Moibi JA, Greene SR, Young R, Trucco M, Gao Z, Matschinsky FM, Deng S, Markman JF, Naji A, Wolf BA: Rapamycin has a deleterious effect on MIN-6 cells and rat and human islets. *Diabetes* 52:2731-2739, 2003

73. Hyder A, Laue C, Schrezenmeir J: Effect of the immunosuppressive regime of Edmonton protocol on the long-term in vitro insulin secretion from islets of two different species and age categories. *Toxicol In Vitro* 19:541-546, 2005

74. Zhang N, Su D, Qu S, Tse T, Bottino R, Balamurugan AN, Xu J, Bromberg JS, Dong HH: Sirolimus is associated with reduced islet engraftment and impaired beta-cell function. *Diabetes* 55:2429-2436, 2006

75. Teutonico A, Schena PF, Di Paolo S: Glucose metabolism in renal transplant recipients: effect of calcineurin inhibitor withdrawal and conversion to sirolimus. *J Am Soc Nephrol* 16:3128-3135, 2005

76. Larsen JL, Bennett RG, Burkman T, Ramirez AL, Yamamoto S, Gulizia J, Radio S, Hamel FG: Tacrolimus and sirolimus cause insulin resistance in normal sprague dawley rats. *Transplantation* 82:466-470, 2006

77. Di Paolo S, Teutonico A, Leogrande D, Capobianco C, Schena PF: Chronic inhibition of mammalian target of rapamycin signaling downregulates insulin receptor substrates 1 and 2 and AKT activation: A crossroad between cancer and diabetes? *J Am Soc Nephrol* 17:2236-2244, 2006

78. Lopez-Talavera JC, Garcia-Ocana A, Sipula I, Takane KK, Cozar-Castellano I, Stewart AF: Hepatocyte growth factor gene therapy for pancreatic islets in diabetes: reducing the minimal islet transplant mass required in a glucocorticoid-free rat model of allogeneic portal vein islet transplantation. *Endocrinology* 145:467-474, 2004

79. Shapiro AM, Suarez-Pinzon WL, Power R, Rabinovitch A: Combination therapy with low dose sirolimus and tacrolimus is synergistic in preventing spontaneous and recurrent autoimmune diabetes in non-obese diabetic mice. *Diabetologia* 45:224-230, 2002

80. Menger MD, Jaeger S, Walter P, Feifel G, Hammersen F, Messmer K: Angiogenesis and hemodynamics of microvasculature of transplanted islets of Langerhans. *Diabetes* 38 Suppl 1:199-201, 1989

81. Vajkoczy P, Menger MD, Simpson E, Messmer K: Angiogenesis and vascularization of murine pancreatic islet isografts. *Transplantation* 60:123-127, 1995

82. Nyqvist D, Kohler M, Wahlstedt H, Berggren PO: Donor islet endothelial cells participate in formation of functional vessels within pancreatic islet grafts. *Diabetes* 54:2287-2293, 2005

83. Linn T, Schneider K, Hammes HP, Preissner KT, Brandhorst H, Morgenstern E, Kiefer F, Bretzel RG: Angiogenic capacity of endothelial cells in islets of Langerhans. *Faseb J* 17:881-883, 2003

84. Brissova M, Fowler M, Wiebe P, Shostak A, Shiota M, Radhika A, Lin PC, Gannon M, Powers AC: Intraislet endothelial cells contribute to revascularization of transplanted pancreatic islets. *Diabetes* 53:1318-1325, 2004

85. Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, Bruns CJ, Zuelke C, Farkas S, Anthuber M, Jauch KW, Geissler EK: Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. *Nat Med* 8:128-135, 2002

86. Butzal M, Loges S, Schweizer M, Fischer U, Gehling UM, Hossfeld DK, Fiedler W: Rapamycin inhibits proliferation and differentiation of human endothelial progenitor cells in vitro. *Exp Cell Res* 300:65-71, 2004

87. Fukuda D, Sata M, Tanaka K, Nagai R: Potent inhibitory effect of sirolimus on circulating vascular progenitor cells. *Circulation* 111:926-931, 2005

88. Kidszun A, Schneider D, Erb D, Hertl G, Schmidt V, Eckhard M, Preissner KT, Breier G, Bretzel RG, Linn T: Isolated pancreatic islets in three-dimensional matrices are responsive to stimulators and inhibitors of angiogenesis. *Cell Transplant* 15:489-497, 2006

89. Cantaluppi V, Biancone L, Romanazzi GM, Figliolini F, Beltramo S, Ninniri MS, Galimi F, Romagnoli R, Franchello A, Salizzoni M, Perin PC, Ricordi C, Segoloni GP, Camussi G: Antiangiogenic and immunomodulatory effects of rapamycin on islet endothelium: relevance for islet transplantation. *Am J Transplant* 6:2601-2611, 2006

90. Lu X, Schuurman HJ, Borel JF: Effect of rapamycin on islet xenograft survival. *Transplant Proc* 26:1128-1129, 1994

91. Iurlaro M, Vacca A, Minischetti M, Ribatti D, Pellegrino A, Sardanelli A, Giacchetta F, Dammacco F: Antiangiogenesis by cyclosporine. *Exp Hematol* 26:1215-1222, 1998

92. Wilasrusmee C, Yusupov I, Ondocin P, Bruch D, Kittur S, Wilasrusmee S, Kittur DS: Angiocidal effect of Cyclosporin A: a new therapeutic approach for pathogenic angiogenesis. *Int Angiol* 24:372-379, 2005

93. Rooth P, Dawidson I, Lafferty K, Diller K, Armstrong J, Pratt P, Simonsen R, Taljedal IB: Prevention of detrimental effect of cyclosporin A on vascular ingrowth of transplanted pancreatic islets with verapamil. *Diabetes* 38 Suppl 1:202-205, 1989

94. Shi CL, Rooth P, Taljedal IB: The protective effect of verapamil on mouse islets transplanted under the kidney capsule. *Transplantation* 56:1491-1495, 1993

95. Basadonna G, Kakizaki K, Merrell RC: Effect of cyclosporine on established islet autografts. *J Surg Res* 40:450-454, 1986

96. Swenne I, Andersson A: Effect of genetic background on the capacity for islet cell replication in mice. *Diabetologia* 27:464-467, 1984

97. Alejandro R, Feldman EC, Bloom AD, Kenyon NS: Effects of cyclosporin on insulin and C-peptide secretion in healthy beagles. *Diabetes* 38:698-703, 1989

98. Ricordi C, Zeng YJ, Alejandro R, Tzakis A, Venkataramanan R, Fung J, Bereiter D, Mintz DH, Starzl TE: In vivo effect of FK506 on human pancreatic islets. *Transplantation* 52:519-522, 1991

99. Strasser S, Alejandro R, Shapiro ET, Ricordi C, Todo S, Mintz DH: Effect of FK506 on insulin secretion in normal dogs. *Metabolism* 41:64-67, 1992

100. Redmon JB, Olson LK, Armstrong MB, Greene MJ, Robertson RP: Effects of tacrolimus (FK506) on human insulin gene expression, insulin mRNA levels, and insulin secretion in HIT-T15 cells. *J Clin Invest* 98:2786-2793, 1996

101. Drachenberg CB, Klassen DK, Weir MR, Wiland A, Fink JC, Bartlett ST, Cangro CB, Blahut S, Papadimitriou JC: Islet cell damage associated with tacrolimus and cyclosporine: morphological features in pancreas allograft biopsies and clinical correlation. *Transplantation* 68:396-402, 1999

102. Shapiro AM, Gallant HL, Hao EG, Lakey JR, McCready T, Rajotte RV, Yatscoff RW, Kneteman NM: The portal immunosuppressive storm: relevance to islet transplantation? *Ther Drug Monit* 27:35-37, 2005

103. Diamond AS, Gill RG: An essential contribution by IFN-gamma to CD8+ T cell-mediated rejection of pancreatic islet allografts. *J Immunol* 165:247-255, 2000

104. Rabinovitch A, Suarez-Pinzon WL, Sorensen O, Bleackley RC, Power RF: IFN-gamma gene expression in pancreatic islet-infiltrating mononuclear cells correlates with autoimmune diabetes in nonobese diabetic mice. *J Immunol* 154:4874-4882, 1995

105. Thomas HE, Darwiche R, Corbett JA, Kay TW: Interleukin-1 plus gamma-interferon-induced pancreatic beta-cell dysfunction is mediated by beta-cell nitric oxide production. *Diabetes* 51:311-316, 2002

106. Yasunami Y, Kojo S, Kitamura H, Toyofuku A, Satoh M, Nakano M, Nabeyama K, Nakamura Y, Matsuoka N, Ikeda S, Tanaka M, Ono J, Nagata N, Ohara O, Taniguchi M: Valpha14 NK T cell-triggered IFN-gamma production by Gr-1+CD11b+ cells mediates early graft loss of syngeneic transplanted islets. *J Exp Med* 202:913-918, 2005

107. Matos M, Park R, Mathis D, Benoist C: Progression to islet destruction in a cyclophosphamide-induced transgenic model: a microarray overview. *Diabetes* 53:2310-2321, 2004

108. Ivakine EA, Gulban OM, Mortin-Toth SM, Wankiewicz E, Scott C, Spurrell D, Canty A, Danska JS: Molecular genetic analysis of the Idd4 locus implicates the IFN response in type 1 diabetes susceptibility in nonobese diabetic mice. *J Immunol* 176:2976-2990, 2006

109. Reddy S, Bradley J: Immunohistochemical demonstration of nitrotyrosine, a biomarker of oxidative stress, in islet cells of the NOD mouse. *Ann N Y Acad Sci* 1037:199-202, 2004

110. Suarez-Pinzon WL, Mabley JG, Strynadka K, Power RF, Szabo C, Rabinovitch A: An inhibitor of inducible nitric oxide synthase and scavenger of peroxynitrite prevents diabetes development in NOD mice. *J Autoimmun* 16:449-455, 2001

111. Stevens RB, Sutherland DE, Ansite JD, Saxena M, Rossini TJ, Levay-Young BK, Hering BJ, Mills CD: Insulin down-regulates the inducible nitric oxide synthase pathway: nitric oxide as cause and effect of diabetes? *J Immunol* 159:5329-5335, 1997

112. Nikolic B, Takeuchi Y, Leykin I, Fudaba Y, Smith RN, Sykes M: Mixed hematopoietic chimerism allows cure of autoimmune diabetes through allogeneic tolerance and reversal of autoimmunity. *Diabetes* 53:376-383, 2004

113. Suarez-Pinzon W, Korbutt GS, Power R, Hooton J, Rajotte RV, Rabinovitch A: Testicular sertoli cells protect islet beta-cells from autoimmune destruction in NOD mice by a transforming growth factor-beta1-dependent mechanism. *Diabetes* 49:1810-1818, 2000

114. Mottram PL, Murray-Segal LJ, Han W, Maguire J, Stein-Oakley A, Mandel TE: Long-term survival of segmental pancreas isografts in NOD/Lt mice treated with anti-CD4 and anti-CD8 monoclonal antibodies. *Diabetes* 47:1399-1405, 1998

115. Gazda LS, Charlton B, Lafferty KJ: Diabetes results from a late change in the autoimmune response of NOD mice. *J Autoimmun* 10:261-270, 1997

116. Green EA, Flavell RA: Tumor necrosis factor-alpha and the progression of diabetes in non-obese diabetic mice. *Immunol Rev* 169:11-22, 1999

117. Lepault F, Gagnerault MC: Characterization of peripheral regulatory CD4+ T cells that prevent diabetes onset in nonobese diabetic mice. *J Immunol* 164:240-247, 2000

118. Kiener PA, Moran-Davis P, Rankin BM, Wahl AF, Aruffo A, Hollenbaugh D: Stimulation of CD40 with purified soluble gp39 induces proinflammatory responses in human monocytes. *J Immunol* 155:4917-4925, 1995

119. Dechanet J, Grosset C, Taupin JL, Merville P, Banchereau J, Ripoche J, Moreau JF: CD40 ligand stimulates proinflammatory cytokine production by human endothelial cells. *J Immunol* 159:5640-5647, 1997

120. Molano RD, Berney T, Li H, Cattan P, Pileggi A, Vizzardelli C, Kenyon NS, Ricordi C, Burkly LC, Inverardi L: Prolonged islet graft survival in NOD mice by blockade of the CD40-CD154 pathway of T-cell costimulation. *Diabetes* 50:270-276, 2001

121. Kenyon NS, Chatzipetrou M, Masetti M, Ranuncoli A, Oliveira M, Wagner JL, Kirk AD, Harlan DM, Burkly LC, Ricordi C: Long-term survival and function of intrahepatic islet allografts in rhesus monkeys treated with humanized anti-CD154. *Proc Natl Acad Sci U S A* 96:8132-8137, 1999

122. Quesenberry PJ, Zhong S, Wang H, Stewart M: Allogeneic chimerism with low-dose irradiation, antigen presensitization, and costimulator blockade in H-2 mismatched mice. *Blood* 97:557-564, 2001

123. Zhang C, Todorov I, Lin CL, Atkinson M, Kandeel F, Forman S, Zeng D: Elimination of insulitis and augmentation of islet beta cell regeneration via induction of chimerism in overtly diabetic NOD mice. *Proc Natl Acad Sci U S A* 104:2337-2342, 2007

124. Pop SM, Wong CP, Culton DA, Clarke SH, Tisch R: Single cell analysis shows decreasing FoxP3 and TGFbeta1 coexpressing CD4+CD25+ regulatory T cells during autoimmune diabetes. *J Exp Med* 201:1333-1346, 2005

125. Peng Y, Laouar Y, Li MO, Green EA, Flavell RA: TGF-beta regulates in vivo expansion of Foxp3-expressing CD4+CD25+ regulatory T cells responsible for protection against diabetes. *Proc Natl Acad Sci U S A* 101:4572-4577, 2004

126. Gysemans CA, Waer M, Valckx D, Laureys JM, Mihkalsky D, Bouillon R, Mathieu C: Early graft failure of xenogeneic islets in NOD mice is accompanied by high levels of interleukin-1 and low levels of transforming growth factor-beta mRNA in the grafts. *Diabetes* 49:1992-1997, 2000

127. Buhler LH, Spitzer TR, Sykes M, Sachs DH, Delmonico FL, Tolkoff-Rubin N, Saidman SL, Sackstein R, McAfee S, Dey B, Colby C, Cosimi AB: Induction of kidney allograft tolerance after transient lymphohematopoietic chimerism in patients with multiple myeloma and end-stage renal disease. *Transplantation* 74:1405-1409, 2002

Summary

Islet transplantation can restore normoglycemia in diabetic patients. However, its application is limited by the high number of islets required to reverse diabetes. As the mass and potency of most human islet preparations are marginal, a large impact on the applicability of islet transplantation is expected from the implementation of strategies that improve engraftment of these islet preparations. The roles of adaptive immunity and immunosuppression on islet engraftment are not well studied. Therefore, the effects of currently used immunosuppression with antilymphocyte serum, tacrolimus and sirolimus on islet engraftment were separated from their impact on immunity and diabetes reversal rates were compared after islet allotransplantation in immunosuppressed and chimeric, non-immunosuppressed NOD mice. Both strategies prevented rejection of islet allografts and reduced the frequency of donor-specific, IFN-γ-secreting T cells. However, in order to achieve stable diabetes reversal with a probability of about 80% after islet allotransplantation in diabetic NOD mice, a fourfold higher islet mass (400 islets) was required in immunosuppressed recipients, compared with non-immunosuppressed recipients made tolerant pretransplant by induction of mixed hematopoietic chimerism (100 islets). The failure of immunosuppressed mice to become normoglycemic after low-dose islet transplantation primarily resulted from the inhibitory effects of tacrolimus combined with sirolimus on islet engraftment and not from the inhibitory effects of this combination on islet graft function. These data suggest that immunotherapeutic strategies that control adaptive immunity without interfering with islet neovascularization or other processes critical to islet engraftment are likely to improve the success rate of marginal mass human islet allografts.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die angegebenen Hilfsmittel benutzt habe.

Die Dissertation ist bisher keiner anderen Fakultät vorgelegt worden.

Ich erkläre, dass ich bisher kein Promotionsverfahren erfolglos beendet habe und dass eine Aberkennung eines bereits erworbenen Doktorgrades nicht vorliegt.

Boston, 15. Juni 2007 Unterschrift

L E B E N S L A U F

PERSÖNLICHE INFORMATIONEN

- Name: Hannes Kalscheuer
- Addresse: 500F Falls Boulevard, Apt. #6222
  Quincy, MA 02169, USA
- Geburtsdatum: 21. Mai 1978
- Geburtsort: Braunschweig
- Familienstand: ledig

SCHULBILDUNG

- August 1984 – Juli 1988: Grundschule Braunschweig-Querum
- August 1988 – April 1989: Orientierungsschule Braunschweig
- April 1989 - Juli 1990: Thomas-Mann-Schule Northeim
- August 1990 - Juni 1997: Gymnasium Corvinianum Northeim
- 09. Juni 1997: Abitur am Gymnasium Corvinianum, Northeim, Auszeichnung für besonderes Engagement im Fachbereich Musik

STUDIUM

- Oktober 1997 - Mai 2005 Ernst-Moritz-Arndt Universität Greifswald
  21. September 2000: Physikum
  20. September 2000: Erstes Staatsexamen
  02. März 2004: Zweites Staatsexamen
  24. Mai 2005: Drittes Staatsexamen, Abschluss Ärztliche Prüfung mit Gesamtnote „gut“

- Oktober 2000 - April 2002 University of Minnesota, Minneapolis, USA
  Forschungs- und Doktorandenaufenthalt bei Prof. B.J. Hering im Diabetes Institute for Immunology and Transplantation, Minneapolis

- April - Juni 2004 University of Wisconsin, Madison, USA
  Chirurgie-Tertial in Thorax- and Transplantations-Chirurgie

- Juni - August 2004 University of Minnesota, Minneapolis, USA
  Chirurgie-Tertial in Allgemein- und Herz-Chirurgie

- August – November 2004 Spital Saanen, Universität Bern, Schweiz
  Innere Medizin Tertial

- November 2004 -März 2005 Ernst-Moritz-Arndt Universität Greifswald
  Anästhesie-Tertial am Universitätskrankenhaus Greifswald

BERUFSERFAHRUNG

- Juli 2005 – Dezember 2006 Assistenzarzt im Spital Saanen, Schweiz, Lehrkrankenhaus Universität Bern
- Seit Mai 2007 Wissenschaftlicher Mitarbeiter am Transplantation Biology Research Center, Harvard University, Boston

PUBLIKATIONEN

- Wu T, Sozen H, Luo B, Heuss N, **Kalscheuer H**, Lan P, Sutherland DE, Hering BJ, Guo Z. "Rapamycin and T cell costimulatory blockade as post-transplant treatment promote fully MHC-mismatched allogeneic bone marrow engraftment under irradiation-free conditioning therapy." **Bone Marrow Transplantation**, 2002; 29 (12): 949-56.

- Guo Z, Wu T, Sozen H, Pan Y, Heuss N, **Kalscheuer H,** Sutherland, DE, Blazar BR, Hering BJ. "A substantial level of donor hematopoietic chimerism is required to protect donor-specific islet grafts in diabetic nod mice." **Transplantation**, 2003; 75 (7): 909-15.

- Pan Y, Luo B, Sozen H, **Kalscheuer H**, Blazar BR, Sutherland DE, Hering BJ, Guo Z. "Blockade of the CD40/CD154 pathway enhances T-cell-depleted allogeneic bone marrow engraftment under nonmyeloablative and irradiation-free conditioning therapy." **Transplantation**, 2003; 76 (1): 216-24.

- **Kalscheuer H**, Liu B, Nakano M, Pan Y, Wu T, Sozen H, Luo B, Heuss N, Clemmings S, Lucido M, Ingulli E, Kirchhof N, Guo Z, Sutherland DE, Hering BJ "A four-fold lower islet mass is sufficient to restore normoglycemia in non-immunosuppressed NOD mice made tolerant pretransplant compared with recipients immunosuppressed with anti-lymphocyte serum, tacrolimus and sirolimus."

  *Submitted to **Transplantation***

VORTRÄGE

- 5.-10. September 2004, XX. International Congress of The Transplantation Society in Wien

  "A Low Donor Islet Mass is Sufficient to Restore Normoglycemia in Diabetic NOD Mice after Pretransplant Induction of a Substantial Level of Donor Chimerism"

FORTBILDUNGEN

- 02.-03. März 2006 Grundkurs Abdomen-Ultraschall, Zweisimmen, Schweiz
- 28.-29. April 2006 Mayo Clinic Review Kurs in klinischer Kardiologie, Zürich, Schweiz
- 20.-27. Mai 2006 Notarzt-Kurs bei der 13. Woche der Notfallmedizin, Langeoog
- 18.-23. Sept. 2006 Grosshadener Kompaktkurs in Endokrinologie und Stoffwechsel, Ludwig-Maximilians-Universität München

SPRACHKENNTNISSE

- Englisch
- Juli 2000: Cambridge Certificate of Medical English
- 1997: Grosses Latinum

BESONDERE INTERESSEN

- Intensive Beschäftigung mit klassischer Musik: Cellounterricht mit 6 Jahren, mehrmalige Teilnahmen an dem Wettbewerb „Jugend musiziert“, sowie Aufführungen mehrerer Solo-Konzerte, u.a. mit dem Göttinger Sinfonie Orchester, dem Northeimer Volkshochschul-Orchester und dem Schulorchester „Capella Corviniensis“ des Gymnasiums Corvinianum Northeim. Mitwirkung in verschiedenen Orchestern, u.a. Universitäts- sowie Kammerorchester der Universität Greifswald, Campus Orchestra der University of Minnesota, Sinfonie- und Kammerorchester des Gymnasium Corvinianums Northeim.
- Mitwirkung bei der Oper „Don Quixote“ von Henry Purcell am Jungen Theater Göttingen; Premiere: 19. Juli 1997
- Mehrmalige Betreuung des Northeimer Nachwuchs-Orchesters bei Studienwochen zwecks Einstudierung von Konzert-Programmen
- Tennis, Skifahren, Literatur

Boston, 15. Juni 2007

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