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EXPERIMENTAL ISLET TRANSPLANTATION

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To my Family
Iam lucky to have you both my parents
Brother Mohammed and sisters Rawya and Sara you were encouraging me with your feelings and love
Dear husband Montasir, you did more than what I needed from you in the whole PhD
I love you all

ABSTRACT

Pancreatic islet transplantation is a promising treatment modality for patients with insulin-dependent diabetes. Besides whole pancreas transplantation, it is the only treatment that can make patients normoglycemic without risking episodes of hypoglycemia. It can also prevent, slow down and even reverse the development of secondary complications to diabetes. Compared to whole pancreas transplantation, islet transplantation is much less invasive and may also be used in patients with a high surgical risk profile, but clinical outcome data are so far better for whole pancreas transplantation. However, graft survival necessitates life-long immunosuppression and for islet transplantation more than one donor is usually needed. There is therefore a need for more specific immunosuppression with less side effects as well as methods by which the donor pool can be expanded.

In this project we have assessed the capability of costimulation blockade, *i.e.* blocking the second signal of T lymphocyte activation, to prevent rejection of allogeneic (between individuals) and xenogeneic (between species) islet grafts, in particular when transplanted to recipients already sensitized to the graft. We have shown that a triple costimulation blockade regimen with anti-CD154 antibodies, CTLA4Ig and anti-LFA-1 antibodies could not prolong survival of islet allografts when transplanted under the kidney capsule of sensitized C57BL/6 mice. Either induced antibodies or memory T cells may be responsible for this inability of conventional costimulation blockade to prolong graft survival in sensitized animals. We tried to resolve this question in a rat-to-mouse xenotransplantation model, in which immune or naïve serum was injected intraperitoneal at the time of islet transplantation. Again, the recipient animals were given costimulation blockade. The immune serum had no negative impact on the grafts immediately (within 96 hours) post-transplantation or on the graft survival long-term in mice receiving costimulation blockade. These results suggest that preformed antibodies are not the main cause for graft rejection in sensitized recipients treated by costimulation blockade.

In the animal transplantation models used, streptozotocin or alloxan is used to induce diabetes through their toxic effects on pancreatic β-cells. It has been reported that these drugs are also toxic for other cells and tissues, including cells of the immune system. Therefore, we compared recipients given streptozotocin or alloxan for diabetes induction with regard to graft survival times, spleen size and toxic effects on leukemic cells *in vitro*. We conclude that streptozotocin is more toxic on immune cells than alloxan, and may therefore not be a suitable agent for diabetes induction in transplantation models assessing different immunosuppressive protocols. Further, we showed that the erythropoietin analogue, pyroglutamate helix B surface peptide (ARA 290) could protect islets from apoptosis when exposed to pro-inflammatory cytokines in vitro, while no clear effect was seen on graft survival when injected into the recipients. Further studies are needed on this potential islet-protective agent.

In conclusion, islet transplantation holds great promise for the future as a treatment modality for insulin-dependent diabetes. However, further research is needed in order to find optimal immunosuppressive protocols with acceptable side effects that can promote long term graft survival. Costimulation blockade may be such a modality provided memory T cell activation can be perturbed and tolerance induced also in sensitized recipients

LIST OF PUBLICATIONS

I. **Randa A. Hadi Diab**, Takashi Iwata, Matthias Corbascio, Annika Tibell, Henrik Ekberg, Jan Holgersson, and Makiko Kumagai-Braesch. **Effect of triple costimulation blockade on islet allograft survival in sensitized mice.** *Transplantation Proceedings, 42, 2109-2111 (2010)*

II. **Randa A. Hadi Diab**, Moustapha Hassan, Annika Tibell, Jan Holgersson and Makiko Kumagai-Braesch. **Rat islets are not rejected by anti-islet antibodies in mice treated with costimulation blockade.** *Xenotransplantation, doi: 10.1111/xen. 12103 (2014).*

III. Yu Saito, Jesper Walmo, Tohru Takahashi, **Randa A. Hadi Diab,** Anthony Cerami, Claes-Göran Östenson, Torbjörn Lundgren, and Makiko Kumagai-Braesch. **A non-hematopoietic erythropoietin analogue, ARA 290, protects rat islets from cytokine-induced apoptosis.** *Manuscript.*

IV. **Randa A. Hadi Diab**, Mona Fares, Manuchehr Abedi-Valugerdi, Makiko Kumagai-Braesch, Jan Holgersson, and Moustapha Hassan. **Toxicological and immunological effects of Streptozotocin and Alloxan *in vitro* and *in vivo* - a comparative study.** *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

ALX	Alloxan
Ab	Antibodies
AUC	Area under the curve
ConA	Concanavalin A
dsDNA	Double stranded DNA
DSA	Donor-specific antibodies
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-Linked ImmunoSpot
EPO	Erythropoietin
EPO-R	Erythropoietin receptor
FBS	Fetal bovine serum
GTKO	α1,3galactosyltransferase gene knock-out
IC50	Half maximal inhibitory concentration
i.p.	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance test
IL-2	Interleukin 2
INF-γ	Interferon-γ
i.v.	Intravenous
IBMIR	Instant blood-mediated inflammatory reaction
ICC	Islet-like cell cluster
KRBB	Krebs-Ringer bicarbonate buffer
MCF	Mean channel fluorescence
MNC	Mononuclear cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OGTT	Oral glucose tolerance test
PBS	Phosphate buffered saline
PMN	Polymorphonuclear
POD	Post-operative day
SGIS	Statistical glucose-stimulated insulin secretion
STZ	Streptozotocin
TNF- α	Tumor necrosis factor
T1DM	Type 1 diabetes mellitus
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
Tx	Transplantation

1 INTRODUCTION

1.1 DIABETES MELLITUS

Type 1 diabetes mellitus (T1DM) is an autoimmune disease that results in the permanent destruction of insulin-producing beta cells of the pancreas. Long-standing diabetes with poor glucose control may lead to complications like retinopathy, nephropathy, neuropathy, vasculopathy and heart disease (myocardial infarction, stroke and peripheral vascular disease)[1]. T1DM affects about 347 million people around the world according to WHO statistics. Formerly known as childhood, juvenile or insulin-dependent diabetes, the cause of type 1 diabetes is still not fully understood. Some scientists have suggested that type 1 diabetes could be a virally-induced autoimmune response. It is related to life style factors and genetics [2]. Pancreatic cancer and other diseases of the pancreas can also cause diabetes, as can certain birth defects [3, 4]. Type 2 diabetes mellitus (T2DM) is the second type of diabetes and the most common form as about 90% of all diabetics has this form. T2DM is a multifactorial disease, and both environmental and genetic factors are likely to influence disease progression [5]. It is a common disorder characterized by a dysregulation of the glucose and lipid metabolism, and is often part of a metabolic syndrome including high blood pressure, obesity and T2DM [5]. T2DM occurs when peripheral tissues become resistant to the effects of insulin, do not make enough insulin or both [6]. Sulfonylureas and biguanides are examples of drugs used for the treatment of T2DM [7]. Further, patients are advised to do regular exercise and to keep optimal food regimens in addition to the medication. Despite this, T2DM patients may become dependent on insulin injections and may develop complications similar to those seen in T1DM patients [7].

1.1.1 Treatment of type 1 diabetes

Insulin replacement therapy by intermittent subcutaneous injections or by way of an insulin pump, along with dietary management, typically including tracking carbohydrate intake as well as careful monitoring of blood glucose levels using various glucose measurement devices, is usually sufficient to keep blood glucose levels under control [1, 8]. However, despite careful control of blood glucose levels, the patient may sometimes experience life-threatening episodes of hyper- or hypoglycemia. Similarly, even though strict control of blood glucose levels by tightly regulated insulin regimens has decreased the incidence of complications, many patients with long-standing T1DM develop retinopathy, neuropathy, nephropathy, vasculopathy and heart disease [1] . Transplantation (Tx) of whole pancreas and pancreatic islets on the other hand are procedures that can not only be used to treat T1DM, but can also reverse it [9, 10]

1.2 THE PANCREAS

The pancreas is a glandular organ of fleshy texture, and is a part of the digestive and endocrine systems of vertebrates. It is the second largest gland in the human body.

1.2.1 Anatomy of the pancreas

Pancreas is located in the posterior abdominal wall, behind the stomach and it is comprised of the head (the broadest part attached to the concavity of the duodenum - the first section of the small intestine), the uncinate process (constricted part between the head and the body), the body (running behind the stomach) and the tail (stretching towards the spleen) (Fig. 1).

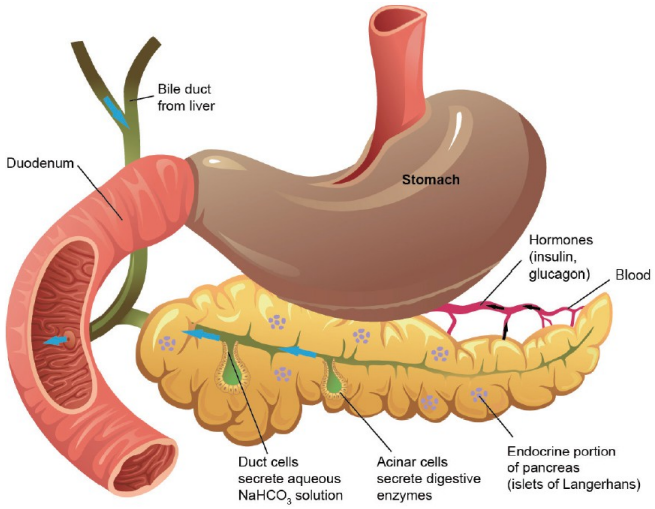


Figure 1 Anatomy of the pancreas

1.2.2 Histology of the pancreas

Under the microscope, stained section of the pancreas shows two different types of parenchymal tissue. Islets of Langerhans are the lightly staining cluster of cells, which produce hormones responsible for the endocrine functions of the pancreas. Darker staining cells form acini connected to ducts. Acinar cells belong to the exocrine pancreas and secrete digestive enzymes into the gut via a system of ducts (Fig. 2).

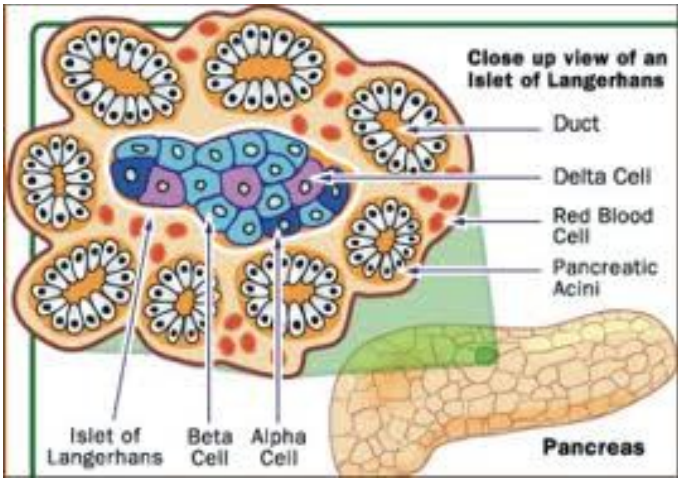


Figure 2 Islets of Langerhans

1.2.3 Physiology of the pancreas

Pancreas is a mixed organ (endocrine and exocrine functions), about 80% of the islets of Langerhans consists of beta cells, which secret insulin to reduce the glucose level in the blood avoiding hyperglycemia [11]. Glucagon, the negative regulator of insulin, is secreted from α cells and stimulates glycogenolysis and gluconeogenesis in the liver.

1.3 WHOLE PANCREAS AND PANCREATIC ISLET TRANSPLANTATION

Whole pancreas transplantation was first attempted in 1966 by the Minnesota surgeons William Kelly and Richard Lillehei [12]. Since then >42,000 pancreas transplants have been reported to the International Pancreas Transplant Registry [13]. With new immunosuppression and improved surgical techniques, 1-year post-transplant patient and graft survival rates are now >95% and approaching 85%, respectively. Pancreas transplantation is an option for patients with T1DM, some patients with T2DM, and for patients with diabetes caused by surgical removal of the pancreas. It restores euglycaemia and, in contrast to insulin-replacement therapy, without the risk of inducing hypoglycaemia [13]. In addition, it can prevent, halt or even reverse the secondary complications of diabetes mellitus. In fact, whole pancreas transplantation is considered the standard of care for patients with diabetes mellitus and uraemia [13]. However, pancreas transplantation is major surgery and technical complications are still the most common reason for graft loss following simultaneous pancreas and kidney transplant, pancreas after kidney transplant and pancreas transplant alone [13].

Islet transplantation is accomplished by injection through the portal vein of islets isolated by collagenase digestion of the pancreatic gland using for example the Ricordi protocol for islet isolation [10, 14]. Because islet transplantation is less invasive than whole pancreas transplantation it is associated with fewer surgical complications [15]. Even though the fraction of insulin-free patients following islet transplantation has increased in recent years (27% at 3 years if transplanted between 1999-2002 to 44% at 3 years if transplanted between

2007 and 2010) [14], long-term outcomes are typically better for whole pancreas transplantation than islet transplantation [13]. Transplantation centers performing both procedures tend to reserve islet transplants for patients with a high surgical risk, while pancreas transplantation is offered patients with a low surgical risk. A clear disadvantage with islet transplantation is that in many cases glands from several donors are needed to obtain enough islets to accomplish insulin-independence. Further, the primary goal of islet transplantation is currently not to achieve insulin-independence, but to reduce the incidence and severity of hypoglycemic events and in exogenous insulin needed [14].

1.3.1 Clinical islet transplantation

Clinical islet transplantation offers the patient intact islets of Langerhans and their physiological ability to control blood glucose levels in a very accurate manner, provided enough islets are injected, engrafting and surviving the host's immune attack. A considerable drawback is that recipients of islets, as with recipients of all allogeneic tissues, will need life-long immunosuppression. Watson Williams was an English surgeon who transplanted sheep pancreas tissue to a 15 years old boy in 1893 [10]. The boy died a few days later from recurring diabetes and complications thereof ([10] This was the first islet xenotransplantation of that time. In 1977, Najarian injected about 50,000 impure islets into the peritoneal cavity of a diabetic patient, and as a result there was a decrease in the blood glucose levels [16]. The report by the research group in Edmonton in July 2000 of 7 consecutive patients that became insulin-independent after islet transplantation was considered a big breakthrough [14]. Improvements to the immunosuppressive protocol, in which steroids were left out and IL-2 receptor blockers were included, and the fact that islets from more than one donor were given, have been put forward as an explanation for the improved results [14]. Even though insulin independence was as low as 10% five years post-transplantation in the cohort of patients done under this protocol, glucose instability and problems with hypoglycemia were reduced [17]. As described above, further improvement of the protocols for islet transplantation has increased the fraction of patients that are independent of insulin three years post-Tx to 44% (transplanted 2007-2010) from 27% (transplanted between 1999 and 2002) [18]. Considering the mere prevalence of diabetes patients that could be eligible for islet transplantation, the fact that most recipients need islets from more than one donor in order to become insulin-independent is a huge problem [19-21] Again, improvements to the protocol may solve this problem. The Minneapolis group recently reported on a group of eight patients that became insulin-independent following transplantation of islets from a single donor [20]. Immunosuppressive drugs are needed for islet allograft survival, but are associated with side effects, including but not limited to a general drug toxicity (*e.g.* nephrotoxicity by calcineurin inhibitors), the risk of tumor development or to suffer from infection (bacterial, viral or fungal infection) [14, 22]. Therefore, there is a need for more specific immunosuppressive drugs, which are associated with fewer side effects. We have in

our models evaluated the effect of blocking co-stimulation pathways using biotherapeutic drugs like antibodies and fusion proteins.

1.4 XENOTRANSPLANTATION

The chronic organ shortage has led the research community to explore the possibility of using other species as organ-, tissue- or cell donors in human transplantation, so called xenotransplantation. The use of pancreatic tissue from another species may be an alternative solution for the lack of human donors in pancreatic islet transplantation [23]. Anatomically and physiologically the organs of pigs are similar to those of human organs and it is easy to breed pigs [24-26]. Small-scale clinical safety trials have been performed in patients with diabetes and Parkinson's disease. These patients did not experience any symptomatic relief, and the immunological rejections are left to be solved before xenografting can be used clinically on a routine basis. The most immediate rejection of vascularized organ xenografts is the hyperacute rejection (HAR) caused by preformed, natural anti-α-Gal Abs in the recipient species reacting with α-Gal antigens on the endothelium in donor organs. This interaction leads to complement and endothelial cell activation, formation of microthrombi and microhemorrhagies, cessation of blood flow, and eventually rejection. With the engineering of pigs lacking the α-Gal epitope, HAR can now be avoided. However, even though HAR can be prevented, discordant organ xenografts are usually lost in an acute vascular rejection (AVR) hours to days post-transplantation. It is histopathologically characterized by endothelial cell (EC) swelling, focal ischemia, a diffuse microvascular thrombosis with fibrin deposition and a cellular infiltrate, dominated by monocytes/macrophages, natural killer (NK) cells and neutrophils. EC activation will promote leukocyte recruitment, platelet aggregation and loss of thromboregulation, all hallmarks of AVR. AVR triggering events are still controversial, but induced anti-graft Abs and molecular species incompatibilities are believed to be important. Islet xenograft rejection is discussed below. Besides rejections, infections from pig to man is also a potential obstacle for clinical xenotransplantation. However, pigs can be bread in a clean environment to reduce the risk of pathogen transmission. Porcine endogenous retroviruses (PERV) are inherited because they are integrated in the host's genome, and one has feared that these viruses may become activated following transplantation, infect human cells and thereafter spread in the human population [27]. Even though these viruses can infect human cells *in vitro*, there is no evidence in the literature for infection of humans following exposure to pig tissue *in vivo* [28, 29]. In addition, the ethics of using animal tissue or cells for transplantation should be discussed and assessed if it is acceptable in the community in the context of religious beliefs and animal rights.

1.4.1 Islet xenotransplantation

It has been shown that pig islets can reverse hyperglycemia and maintain normoglycemia in diabetic nonhuman primates for more than six months [30-34]. Different immunosuppressive strategies have been taken to accomplish this. Encapsulation without systemic immunosuppression [31, 35] or extensive systemic immunosuppression [35] was used to prevent rejection. Remaining challenges precluding clinical islet xenotransplantation include, but are not limited to, the instant blood-mediated inflammatory reaction (IBMIR) [36, 37] and the strong CD4+ T-cell/macrophage-mediated rejection [38, 39].

Already in the beginning of the 1990's, Groth and coworkers transplanted fetal porcine islet-like cell clusters into patients with T1DM. Pig C-peptide was detected in the urine beyond 300 days and pig islet cells were detected in biopsies in patients receiving combined fetal pig islet and human kidney transplants [40]. Since then two limited clinical trials with pig-to-human islet transplantation have been performed in Mexico and New Zealand, respectively. Even though most data suggests that the pig islets were rapidly lost in these clinical studies, it has been shown that pig islets can reverse hyperglycemia and maintain normoglycemia in diabetic nonhuman primates for more than six months. Different immunosuppressive strategies have been taken to accomplish this. Encapsulation without systemic immunosuppression [31, 35] or extensive systemic immunosuppression was used to prevent rejection. Remaining challenges precluding clinical islet xenotransplantation include, but are not limited to, the instant blood-mediated inflammatory reaction (IBMIR) and the strong CD4+ T-cell/macrophage-mediated rejection.

1.5 THE IMMUNE SYSTEM

The immune system consists of a collection of cells and tissues that defend us against invading microorganisms, other foreign bodies and tumors. Functionally, it protects us against infectious agents like bacteria, viruses, fungi and parasites. There are two types of immunity, innate and adaptive immunity. The innate immune response is responsible for an immediate response against microbial invasion. It relies on several factors, including the epithelial barriers, factors, *e.g.* the complement factors, produced at the epithelial surfaces, natural antibodies and cellular elements such as neutrophilic granulocytes, monocytes/macrophages and natural killer cells [41]. The adaptive immune response is slower, but more specific. It evolves as a specific response to microbes or foreign cells, including transplanted allogeneic cells [41]. T- and B-lymphocytes are the key cellular elements of an adaptive immune response. They carry antigen-specific receptors that bind peptides derived from protein-based antigens in case of the T-cell receptor and conformational epitopes in case of the B-cell receptor [41]. Besides the cellular elements, specific antibodies of different classes are important players of the adaptive immune system.

1.5.1 Islet allo- and xenograft rejection

Islet allografts express donor MHC molecules, which may be recognized by the host's T-cells by two pathways. This allorecognition can be direct, *i.e.* recognition of donor MHC on donor antigen presenting cells (APC), or indirect, *i.e.* donor alloantigens are internalized by recipient APC, processed, and presented as peptides by recipient MHC molecules. T-cells triggered by direct recognition are of high frequency, while the latter is a low-frequency response. Both pathways are important during allograft rejection and it is thought that the direct pathway is responsible for acute rejection and the indirect pathway for chronic rejection, which occurs later after transplantation [42]. Early adoptive transfer experiments showed that primary CD4+, but not primary CD8+, T cells were not only necessary for rejection of islet allografts but were also sufficient to mediate rejection [43]. It was speculated that CD4+ T cells could reject islet allografts by a direct cytolytic mechanism or IFN-γ secretion, or by indirectly activating B-cells producing graft-specific Abs that could activate complement or mediate an antibody-dependent cell-mediated cytotoxic effect on the graft [43]. In light of this it is interesting to note that the presence of HLA Abs before transplantation was shown to be associated with reduced graft survival after clinical islet allotransplantation. Further, the potential return of the autoimmune disease in grafted allogeneic islets has to be considered, and optimal immunosuppression for both autoimmune as well as allogeneic rejection should be used [44].

The kinetics and mechanisms of cellular xenograft rejection differs from that of organ xenograft rejection, mainly in that HAR does not occur due to the absence of a vascular network at the time of transplantation. Host immune cells have to traverse host, and not xenogeneic, endothelium to reach grafted cells. The rejection of islets xenografts in rodents is completed by day 10 and has been shown to be a $CD4^+$ T cell-dependent process. Macrophages activated by $CD4^+$ T cells are believed to be direct effectors of cellular xenograft rejection and specific depletion of macrophages delays cellular infiltration and rejection of adult porcine islets in mice. In addition, it was recently demonstrated that macrophages activated by $CD4^+$ T-cells, were capable of both recognition and rejection of pancreatic islet xenografts. The role of natural anti-Gal Abs in cellular xenograft rejection is still debated. However, T cell activation and the characteristics of antigen presentation can be expected to be of similar importance in xenogeneic cell and organ rejection. Islet cells transplanted into immunocompetent recipients are rapidly lost due to the reactions described above, leading to apoptotic or lytic death of islet cells. The capacity to engineer pancreatic islets before transplantation with genes protecting from apoptosis or interfering with immune rejections holds great promise for the future. Thus, systemic co-stimulation blockade can support xenogeneic islet survival long-term with preserved metabolic control in the recipient. This was shown by us in a pig islet-to-naïve C57BL/6 mouse model, and has been shown in primates as described above.

1.5.2 The instant blood-mediated inflammatory reaction

Intra-portal injection is the preferred route of islet administration in clinical islet transplantation. The direct interaction between islets and whole blood has been studied in an ingenious *in vitro* system developed by Bennet, Korsgren, Nilsson and coworkers [45]. They showed that the injection of pig islets into human whole blood led to islet platelet adhesion and coagulation, deposition of complement, leukocyte accumulation, islet damage and as a consequence, insulin release. No immunoglobulin deposition could be detected on the islet surface, indicating that the complement system was activated via the alternative pathway. Injection of human islets into human blood, triggered platelet adhesion, coagulation, but no deposition of complement could be seen, and insulin dumping was milder. Clotting and islet damage were also evident *in vivo* during intraportal injection of pig islets into pigs (allogeneic) or cynomologus monkeys (xenogeneic). The addition of soluble complement receptor-1 (sCR1) and heparin to the blood prior to islet injection inhibited both coagulation and complement activation. Furthermore, leukocyte infiltration and islet morphology was improved; supporting the notion the inhibiting IBMIR will be important to augment islet engraftment. Similar results were observed by addition of a thrombin inhibitor (Melagatoran). Recently, it was shown that tissue factor (TF), which is a physiological trigger of the coagulation cascade, is synthesized and secreted by both α and β cells of islets. Blocking the active site of TF was also effective in order to inhibit IBMIR.

1.6 IMMUNOSUPPRESSIVE TREATMENT IN CLINICAL ISLET TRANSPLANTATION

In the early days of islet transplantation, the immunosuppressive protocols were similar to those used for kidney transplantation and including azathioprine, cyclosporine and corticosteroids [44]. One reason for the improved outcome of islet transplantation using the Edmonton protocol was the modified immunosuppression. In their protocol, immunosuppression was started already prior to transplantation and steroids were excluded [46]. They replaced glucocorticoids with daclizumab, a mAb against the IL-2 receptor and for maintenance therapy they used sirolimus (a target of rapamycin inhibitor) and low-dose tacrolimus [46]. This protocol and slight modifications of it is currently in clinical use. However, going forward the aim is to eliminate calcineurin inhibitors completely from the immunosuppressive regiment. One protocol has added the TNF-a receptor antagonist, etanercept, and the glucagon-like peptide-1 analogue to the Edmonton protocol [46]. Another uses antithymocyte globulin (ATG) and etanercept for induction and cyclosporine and everolimus for maintenance [44].

1.6.1 Co-stimulation blockade

Immune system as mentioned before, is an innate and adaptive immunity, the adaptive immunity is include T cells, these T cells are important in the transplantation field, as they have a response against the foreigners, whether these foreigners are microbes or viruses or even a transplanted cells or tissue. As soon as T cells recognize these species they will act against it and fight it. So we have to think how to block these receptors and then T cells will not be activated against the transplanted agent and the rejection can be reduced or avoided. T cells have many types of receptors and ligands, some are inhibitors and some are activators for T-lymphocytes, CTLA4Ig have been blocked in a mouse model resulting in long survival time for the transplanted islets [47]. In previous studies we showed that CTLA4Ig, anti-CD40L and anti-LFA-1 can lead to a longer survival time for the graft [48]. On paper one and two we used these co stimulations in naïve and sensitized C57BL/6 mice transplanted with rat or BALB/c mouse islets, presence of HLA in allotransplantation can reduce the graft survival [49, 50], and cause hyper acute rejection [51], immediate blood-mediated inflammatory reaction (IBMIR) can also occurs [37].

1.7 IMPROVEMENT OF GRAFT ISLET QUALITY: STUDY OF A NEW CYTOPROTECTIVE DRUG

Islet quality may decrease during their isolation and manipulation, and this may reduce the number of islets obtained from the donor. In clinical islet transplantation using the portal vein route for injection, many islets are destroyed due to the immediate blood mediated reaction (IBMIR; see above) [52]. In this process, coagulation pathways are activated and a severe inflammatory response is induced around the grafts, which can impair function and cause islet apoptosis. Drugs that reduce the inflammatory or pro-thrombotic responses in recipients or increase robustness of islets may improve graft survival and reduce the number of donors needed for each patient.

Erythropoietin (EPO) is a hormone that regulates haematopoiesis by its binding to the erythropoietin receptor (EPO-R). Administration of high doses of EPO is known to show tissue protective effect. Brines *et.al.* [53] found that EPO mediates tissue protection through an erythropoietin and common beta-subunit (CD131) heteroreceptor. The heterodimer EPO-Rs are expressed in a variety of tissues and cells including the small bowel [54], myoblasts [55], neuronal cells [56], kidney cells [57] and cells in pancreatic islets [58].

Recently, an erythropoietin analogue, pyroglutamate helix B surface peptide (ARA 290), without hematopoietic function was developed. It has 11 amino acids and a high specificity for the EPO-R-CD131 heterocomplex [53]. The drug is currently in ongoing or planned phase 2 clinical trials in patients with sarcoidosis, type 2 diabetes and rheumatoid arthritis.

It has been reported that pancreatic islets express EPO-R-CD131 heteroreceptors and that

EPO can improve rat islet cell viability and function when exposed to pro-inflammatory cytokines [58]. Our hypothesis is that ARA 290 treatment may improve islet viability and function during inflammation such as the cytokine-induced inflammation caused by IBMIR or brain death.

1.8 STREPTOZOTOCIN AND ALLOXAN

Streptozotocin (STZ) and alloxan (ALX) are widely used agents for induction of diabetes in experimental animal models of insulin-dependent diabetes, in particular those used for islet transplantation research. They have a cytotoxic effect on pancreatic beta cells, which are killed by necrosis leading to insulin deficiency and diabetes. Because of their similarity to glucose, both agents accumulate in beta cells through uptake via the glucose transporter, GLUT2 [59, 60]. The cytotoxic mechanisms of STZ and ALX are completely different [61]. In the presence of intracellular thiols, e.g. glutathione, ALX generates reactive oxygen species through a cyclic redox reaction [62]. The final product of these reactions is hydroxyl radicals that are finally responsible for beta cell death [63]. In addition, ALX inhibits the beta cell glucose sensor, glucokinase, thereby perturbing glucose-induced insulin secretion [63]. Through its alkylating properties, STZ on the other hand exerts its beta cell-destroying action by modifying macromolecules and fragmenting DNA [63]. Because it also fragments mitochondrial DNA, it perturbs mitochondrial metabolism and thereby glucose-induced insulin secretion [61]. Additional organs, like the kidney and liver, expressing the GLUT2 transporter are also affected by the two drugs, mainly by STZ [64, 65].Despite the fact that both STZ and ALX are used to induce diabetes, significant differences are observed in terms of their effects on the host's immune system [66]. STZ affect adaptive immunity and it has a direct toxicity on lymphocytes, mainly CD8+ T cells and B cells [67]. Rubinstein et al have suggested that the hyperglycemic state in mice mainly affects the immunological memory [68]. Sakowicz-Burkiewicz et al reported that T cells isolated from the spleen of rats with STZ-induced diabetes proliferated less than cells isolated from normal rats when stimulated with mitogen, anti-CD3 or anti-CD28 antibodies [69]. Considering the different effects of ALX and STZ on the immune system [70], islet transplantation models using mice with diabetes induced by the two drugs may confound results of studies on novel immune-modulating treatment regimens [70]. In some cases, islet allografts were accepted without immunosuppressive treatment [70]. Some authors have suggested that STZ is a mild immunosuppressive agent, while ALX is not [67]. However, some reports claim that mice with ALX-induced diabetes can accept islet allografts permanently [70].Even though there are several reports on the effects of STZ and ALX on beta cells [63], few investigations have done a side-by-side comparison of their cytotoxic effects in vitro and their effects on the immune system in vivo [71, 72]. It is important to understand the differences in immune response between non-diabetic control mice and mice with STZ-ALX-induced diabetes. We also investigate their cytotoxic effects on human and murine leukemic cell lines.

2 AIMS

- To investigate the effects of costimulation blockade using anti-CD154 and CTLA4Ig with or without anti-LFA-1 antibodies on allogeneic islet graft survival in sensitized mice

- To investigate the effect of preformed donor-reactive antibodies on rat islet xenograft survival in mice with or without costimulation blockade

- To evaluate the effect of ARA 290 - an erythropoietin analogue - on islet viability and function *in vitro* and in a syngeneic rat islet transplantation model

- To assess and compare the effects of streptozotocin (STZ) and alloxan (ALX) on rejection kinetics, lymphocyte number and function *in vivo*, and cytotoxic potency *in vitro*

3 MATERIALS AND METHODS

3.1 ETHICAL PERMISSION

All animal experiments were approved by the regional committee (Stockholm södra djuretiska nämnd) on animal ethics, S161-08, S163-08, S35-10, S74-11 and S78-11 for papers II and IV, S14-07 and S15-07 for paper Ie, and S84-12 and S17-13 for paper III. The requirements of the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of laboratory animals were followed. The animals were fed a commercial diet with free access to food and water.

3.2 ANIMALS AND TRANSPLANTATION MODELS

A mouse allogeneic transplantation model using C57BL/6 mice as recipients and BALB/c as donors were used in paper I. Recipients were sensitized by transplantation of BALB/c pancreatic islets under the kidney capsule or by intraperitoneal injection.

In paper II, we used a rat-to-mouse islet xenotransplantation model with C5BL/6 mice as recipients and Lewis rats as donors. Recipients were divided into three groups receiving: (i) isotype control Abs; (ii) anti-CD154 and CTLA4Ig; or (iii) anti-CD154, CTLA4Ig, and anti-LFA-1 every second day, day 0–8. At the time of transplantation (Tx), half of the animals in each group received naïve mouse serum and half xenoimmune serum derived from mice previously transplanted with rat islets.

In manuscript III, we used a syngeneic rat transplantation model with male Lewis rats as donors and female Lewis rats as recipients. More islets can be retrieved from male rats and female rats do not increase their body weight as much as male rats.

In manuscript IV, we have compared the effect of STZ and ALX on C57BL/6 mice recipients with regard to body weight, rejection kinetics, spleen size and cytotoxic effects on cell lines *in vitro*.

3.3 INDUCTION OF DIABETES

Alloxan (ALX, 75 mg/ml) or streptozotocin (STZ, 180 mg/ml for a mouse and 55 mg/kg for a rat) was used to induce diabetes. An animal was considered diabetic if its blood glucose level exceeded 20 mM for two or more consecutive days. ALX was used in papers I and IV, and STZ in paper II, III and IV.

3.4 ISLET ISOLATION AND PREPARATION

Islets from male Lewis rats were digested using a collagenase solution in Hanks balanced salt solution and were purified using density gradient centrifugation. Isolated islets were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37ºC in a humidified atmosphere (5% CO2 and 95% air).

Mouse islets were isolated following a procedure similar to that used for rat islet isolation. Collagenase concentration was 1.5 mg/ml, the injection volume was 2 ml/mouse and digestion time was 8 min.

3.5 GENERATION OF IMMUNE MOUSE SERUM AND MOUSE ECIPIENTS SENSITIZED AGAINST DONOR ISLETS

In order to generate mouse serum containing anti-donor islet antibodies (Abs) and sensitized mouse recipients, Lewis rat islets (in paper II) or Balb/c mouse islets (paper I) were transplanted/injected to/in C57BL/6 mice. The mouse serum was collected four weeks after transplantation and the induction of anti-donor Abs was verified by flow cytometry on B cell-depleted splenocytes. Mouse sera that had a reactivity >10 channels above the negative control serum were considered positive. All naïve mice lacked anti-rat Abs of IgG isotype. The appearance of IgG Abs against rat splenocytes was taken as proof of successful sensitization.

3.6 TRANSPLANTATION OF ALLOGENEIC ISLETS TO THE SENSITIZED RECIPIENTS

In paper I, 3-4 days after induction of diabetes (blood glucose >20 mmol/L), 200 Balb/c islets were transplanted under the right kidney capsule of C57BL/6 mice. Recipients were divided into 4 groups: group 0, naïve recipients transplanted with 200 allogeneic islets; group 1, mice receiving isotype control antibodies (human IgG, hamster IgG, and rat IgG2a); group 2, mice treated with anti-CD154 and CTLA4Ig; and group 3, injected with anti-CD154, CTLA4Ig, and anti-LFA-1. Injections were given every second day from day -2 up to day 8. Graft function was estimated by daily monitoring of blood glucose and body weight. Non-fasting blood glucose levels of <10 mmol/L in transplanted recipients reflected functional grafts. Blood glucose levels of >20 mmol/L (360 mg/dL) for >2 consecutive days were considered to be indicative for graft rejection, in which case the mouse was sacrificed.

3.7 RAT ISLET-TO-MOUSE TRANSPLANTATION MODEL: STUDY GROUPS, PROCEDURE AND FOLLOW UP

In paper II, Lewis rat islets (100-150) were transplanted under the left kidney capsule of diabetic C57BL/6 mice. Table 1 summarizes the different study groups. The body weight and blood glucose levels were monitored daily for the first ten days and then once a week if there were no signs of rejection. Rejection was defined as above. If recipients were normoglycemic >3 months, the graft-bearing kidney was explanted to verify reoccurrence of diabetes. Serum samples were collected at the end of the follow-up.

Table 1: Study groups used.

Group	n	Injected serum[a]	Costimulation blockade[b]
1	4	naïve	isotype control Abs[c]
2	5	immune	isotype control Abs
3	5	naïve	CTLA4Ig, anti-CD154 Abs, rat IgG2a[d]
4	6	immune	CTLA4Ig, anti-CD154 Abs, rat IgG2a
5	5	naïve	CTLA4Ig, anti-CD154 Abs, anti-LFA-1 Abs
6	6	immune	CTLA4Ig, anti-CD154 Abs, anti-LFA-1 Abs

[a]One hundred μl naïve or immune serum were injected intraperitoneally (i.p.) at the time of transplantation
[b]isotype control antibodies or costimulation blockade-inducing antibodies/fusion proteins were given i.p on days 0, 2, 4, 6, and 8 after transplantation
[c]Abs, antibodies (human IgG, hamster IgG and rat IgG2a)
[d]rat IgG2a is an isotype control for anti-mouse LFA-1

3.8 INTRAPERITONEAL GLUCOSE TOLERANCE TESTS (IPGTT)

In order to assess islet graft function in paper II, intraperitoneal glucose tolerance tests (IPGTT) were performed on euglycemic recipients at one and four months after transplantation as described [48]. The area under the curve (AUC) was calculated and compared between study groups and to that of healthy controls.

3.9 MORPHOLOGICAL EXAMINATION OF EXPLANTED GRAFTS

Graft-bearing kidneys were removed at the end of the observation period. The grafts with adjacent kidney tissue were fixed, dehydrated and sectioned. Serial sections (4 μm thick) were obtained and stained with hematoxylin and eosin for scoring. The scoring was based on the evaluation of sections taken at four to eight different levels throughout the graft. In order to compare graft mass and the degree of cell infiltration into the graft, each section was scored with regard to the fraction of endocrine cells, and the degree of immune and inflammatory cell infiltration. The scoring was based on cell counting performed by two individuals independently.

Apoptotic cells were detected by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using the DeadEnd™ Fluorometric TUNEL System. One glass slide from each graft containing two sections was used for TUNEL staining. The number of TUNEL-positive cells in two sections (taken at two different levels) of the graft was counted under the fluorescence microscope and the mean number of apoptotic cells were used for scoring.

3.10 APOPTOSIS INDUCTION AND DETECTION

In manuscript III, islet apoptosis was induced by delivering a pro-apoptotic signal with rat-specific cytokines as follows: interleukin (IL)-1β (0.5 ng/mL), interferon (IFN)-γ (100 ng/mL) and tumor necrosis factor (TNF)-α (50 ng/ml) for 6 or 12 hours with or without 100 nM ARA 290.

Apoptosis was assessed using the Caspase-Glo 3/7 Assay. In total, 50-100 islets were transferred to a 1.5 mL eppendorf tube. The islets were pelleted by brief centrifugation, resuspended and sonicated for 10 seconds. The Caspase-Glo 3/7 reagent was added and incubated at room temperature for one hour. The luciferase activity was measured with a luminometer. The luciferase activity was related to the amount of double stranded DNA (dsDNA), as determined by the Quant-iT™ Pico Green® dsDNA Assay Kit, in order to correct for the number of cells. Fluorescence was measured with a fluorometer. Each treatment/assay was based on n=5 replicate samples.

3.11 THE MTT ASSAY FOR ISLET VIABILITY ASSESSMENT

The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay was conducted as previously described. Briefly, 20 μL of MTT solution was added to each well containing 20 islets in 200 μL of RPMI-1640 medium and incubated for 2 hours at 37°C. After incubation, the plate was centrifuged, the incubation medium discarded, and the precipitates dissolved in 200 μL of dimethyl sulfoxide. After further centrifugation, 50 μL of the supernatant was removed for the MTT assay. The absorbance was measured spectrophotometrically at 550 nm.

3.12 STATIC GLUCOSE-STIMULATED INSULIN SECRETION (SGIS) TESTS

In manuscript III, the static glucose-stimulated insulin secretion (SGIS) was assessed. Twenty islets were hand-picked and transferred to 24-well Transwell® plates (8.0 μm pore size membrane) in 1.5 mL of Krebs-Ringer bicarbonate buffer (KRBB) containing 1.67 mM glucose (low glucose KRBB). The islets were incubated for 60 min with 1.5 mL of low glucose KRBB, followed by incubation with high glucose KRBB (glucose 16.7 mM) for another 60 min. The supernatants were collected and the insulin in the supernatants was

quantified using a rat insulin enzyme-linked immunosorbent assay (ELISA) kit. A stimulation index was calculated by dividing the total amount of insulin released from the islets cultured in the high-glucose KRBB by the total amount of insulin released from the islets cultured in the low-glucose KRBB.

3.13 TRANSPLANTATION OF A MARGINAL NUMBER OF ISLETS

To determine the optimal number of islets in manuscript III, preliminary transplantation under the kidney capsule using 600, 500, 400, 300, 250 and 150 islets was performed. The cure rate of the groups that were transplanted with more than 250 islets was 100%, but no groups were cured by transplantation with 150 islets. Therefore, the marginal islet number was determined to be 220. In total, 220 Lewis rat islets were picked and washed once with HBSS and then packed into a 24 GA Venflon™ (BD) using a Hamilton syringe. The islets were placed under the left kidney capsule of diabetic female Lewis rats.

3.14 ARA290 TREATMENT

Recipient rats were randomized into either the vehicle treated control group (n=5) or the ARA 290 group (n=4). On the day of transplantation, ARA 290 (60 μg/kg) was administered intraperitonealy at 0 and 2 hours pre-transplantation and subcutaneously (120 μg/kg) at 6 and 12 hours post-transplantation. The same dose was administered subcutaneously twice a day from postoperative day (POD) 1 to 7 and finally, once a day from POD 8 to 14.

3.15 POST-TRANSPLANT MANAGEMENT OF RECIPIENT ANIMALS

In manuscript III, the non-fasting blood glucose levels and weights of the animals were measured daily for 30 days. Subsequently, graftectomy was performed at 28 POD and the blood glucose levels were followed for another 3 days in order to confirm the recurrence of diabetes. Two of the rats in the control group were discarded because their blood glucose level remained below 20 mM after the graftectomy (n=7 total but it became 5 in final). Several of the kidneys with graft bearing were homogenized in acid-ethanol for insulin extraction. All of the rats were sacrificed 3 days after the graftectomy at 31POD.

3.16 ORAL GLUCOSE TOLERANCE TEST (OGTT)

An oral glucose tolerance test (OGTT) was performed four weeks after transplantation in all the transplanted rats. A total of 1.5 g glucose/kg bodyweight was administered orally to the rats after 16 hours of fasting, and the blood glucose levels were measured at 0, 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes after glucose challenge. The insulin levels in the plasma were measured at 0, 10, 20, 30, 45, and 60 minutes using a rat insulin ELISA kit (Mercodia).

3.17 CELL CULTURE, DETERMINATION OF CYTOTOXICITY AND IC_{50} VALUES

The HL60 cell line was purchased from DSMZ, and the K562 and C1498 cell lines were purchased from ATCC. HL60 and K562 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and C1498 cells were cultured in DMEM medium supplemented with 10 % heat inactivated FBS and 1% penicillin/streptomycin. Cells were cultured in a concentration of 2×10^5 cells/ml and incubated at 37°C in a 95% humidified and 5% CO_2 atmosphere. All experiments were performed on exponentially growing cells in complete medium.

Human and murine cell lines were cultured in triplicate in 96-well plates at a density of $2x10^4$ cells/well in the absence (untreated control) or presence of various concentrations of ALX (20-3000 μg/ml) or STZ (1-3000 μg/ml) for 48h at 37 °C under a humidified atmosphere containing 5% CO_2. Cells incubated in complete media including dH_2O in a final concentration of 0.1% served as control for solvent toxicity and cells incubated in complete medium used as a control for the experiments. The effects of the tested drugs on tumor cell growth or viability were determined employing the MTT assay (Sigma-Aldrich) in accordance with the manufacturer's instructions. The IC_{50} values (drug concentration that induce 50% inhibition of the cell growth) were calculated using the GraphPad Prism 4 program (GraphPad software Inc., CA, USA).

3.18 ISLET TRANSPLANTATION

For manuscript IV, rat or mouse islets were handpicked under the microscope and transplanted under the kidney capsule of recipients with chemically induced diabetes (non-fasting blood glucose >20 mM). Recipient's body weight and blood glucose were measured daily until rejection. Rejection was defined as described above. When grafts were rejected, the mice were sacrificed. The grafts, spleen and serum were harvested.

3.19 PROLIFERATION TESTS

Isolated splenocytes from C57BL/6 mice were used as responder cells and splenocytes from Balb/c mice were used as allogeneic stimulators. Some of C57BL/6 mice were injected with STZ at a dose of 180 mg/kg or ALX 75 mg/ml four days prior to euthanasia. The stimulator cells were irradiated with a 15 Gy dose. Subsequently, 10^5 responder cells/well and 4 x 10^5 stimulator cells/well were mixed to a final volume of 200 μl in RPMI 1640 supplemented with 10% FBS, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol. The proliferation test was performed in triplicate in U-bottomed, 96-well tissue culture plates. Cells were incubated at 37°C in humidified air containing 5% CO_2. Each culture was labeled with 2 μCi ^3H thymidine (Amersham, UK) approximately 20

hours prior to harvest (day 5). [^{3}H] thymidine incorporation was measured using a beta counter (Wallac, Sverige AB, Sweden).

3.20 STATISTICS

Differences between two groups were analyzed by the Mann Whitney U test. If comparisons between three or more groups were performed, the Kruskal-Wallis test with Dunn's multiple comparison tests (GraphPad Prism ver.6) was used. Survival was analyzed by a log-rank (Matel-Cox) test with the Bonferroni post-hoc test for multiple comparisons. A P-value of less than 0.05 was considered statistically significant.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Naïve mice rejected islet allografts between 7 and 29 days (mean 16 ± 8 d), sensitized mice in the control group (group 1) between days 0 and 14 (mean 7 ± 5 d), sensitized mice receiving double (anti-CD154 and CTLA4Ig) costimulation blockade (group 2) between days 4 and 16 (mean 8 ± 4 d), and sensitized mice receiving triple (anti-CD154, CTLA4Ig and anti-LFA-1) costimulation blockade (group 3) between days 4 and 26 (mean 11 ± 7 d). Two naïve mice transplanted with islet allografts and receiving triple therapy accepted their allografts (±180 days; data not shown). Blood glucose and body weight data of individual mice after transplantation is shown in Figure 3. The data of naïve mice that were transplanted with allogeneic islets and treated with costimulation blockade is shown in Figure 3B. The graft function was maintained over 60 days, which was confirmed by graftectomy resulting in recurrence of diabetes.

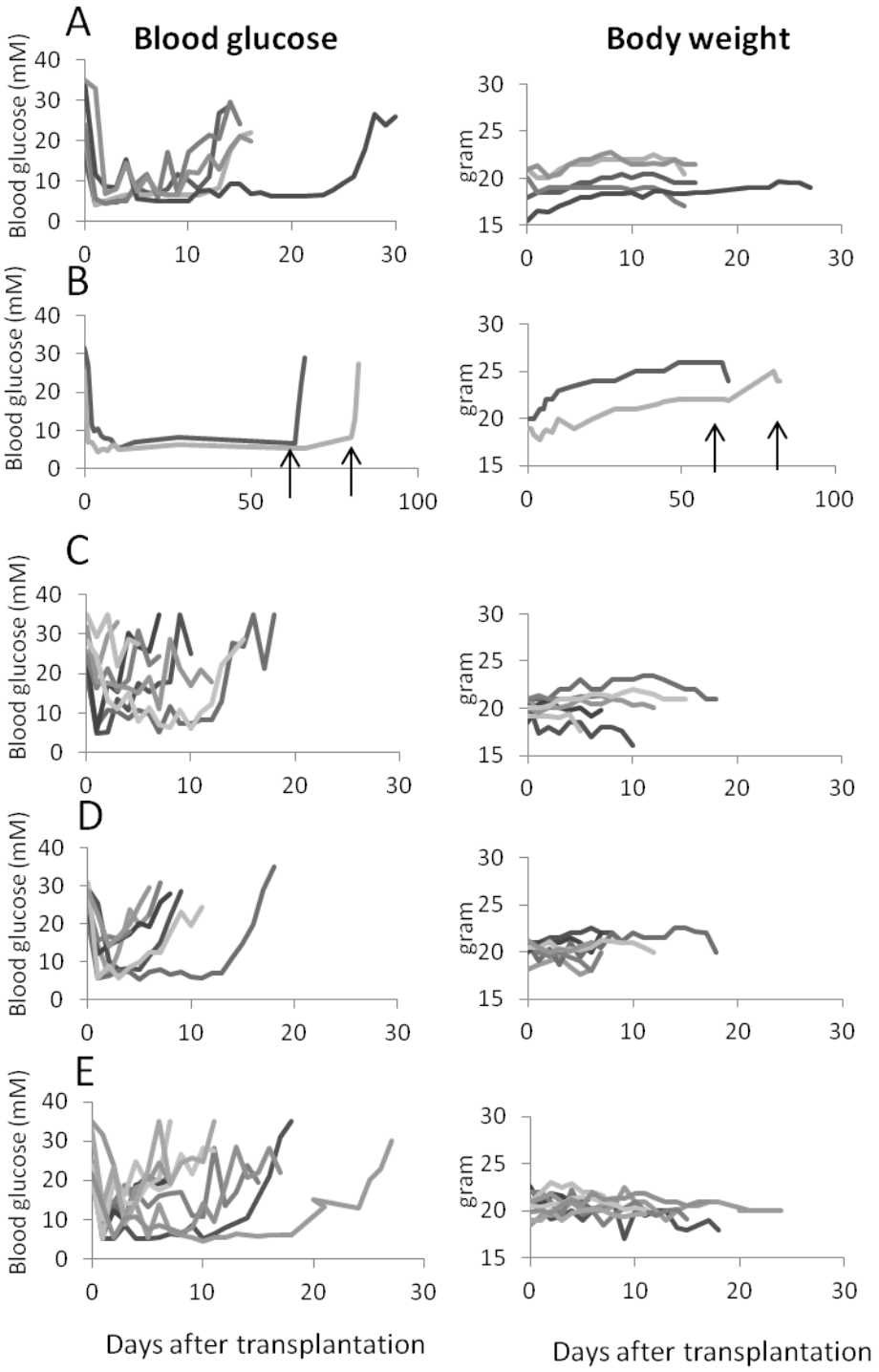

A
Blood glucose
Body weight
Blood glucose (mM)
gram
B
C
D
E
Days after transplantation
Days after transplantation

Figure 3 Blood glucose and body weight after transplantation in each group are shown.
(A) Naïve C57Bl/6 recipients were transplanted with Balb/c islets and isotype control antibodies were injected as described in the materials and methods. Grafts were rejected between 7 and 23 days.
(B) Naïve C57Bl/6 recipients were transplanted with Balb/c islets with costimulation blockade treatment; grey bar shows the results of a mouse treated with CTLA4Ig and anti CD154, dark bar with CTLA4Ig, anti CD154 and anti LFA-1 antibodies. Arrows indicated the graft bearing nephrectomy in order to confirm the graft function.
(C-E) Sensitized mice were transplanted with Balb/c islets and treated with (C) isotype control antibodies, (D) CTLA4Ig and anti CD154 antibodies, and (E) CTLA4Ig, anti CD154 and anti LFA-1 antibodies.

Table 2: Graft Survival after islet allotransplantation

Group	Graft Survival (d)
Naive mice (n=5)	7,14,29,15,14
Sensitized mice treated with isotype control Abs (n=8)	0,0*, 4,5,8,9,13,14
Sensitized mice treated with double costimualtion blockade (n=7)	4,4,6*, 7*, 8,8,16
Sensitized mice treated with triple costimualtion blockade (n=10)	4,5*, 6,7*, 7*, 8,13,14,17,26

*Immunized by intraperitoneal injection of a BALB/c pancreatic digest (including islets); others immunized by transplantation of 200 islets under the kidney capsule.Triple co stimulation blockade could not prolong the graft to survive in the sensitized mice model.

4.1.1 Discussion

Costimulation blockade has been proven useful in order to induce long term graft survival in rodent models. However, it has been difficult to achieve similar efficiency in large animal models. In larger animals and humans, costimulation blockade needs to be complemented with conventional immunosuppressive drugs used for maintenance therapy (*e.g.* rapamycin or MMF) or repeated injections of costimulation blockade agents are necessary [73]. A potential explanation for this is the higher frequency of alloreactive memory cells in larger animals and humans.

Previously, we have shown that double costimulation blockade with CTLA4Ig and anti-CD154 Abs can induce long-term graft survival, and addition of anti-LFA-1 Abs improves graft function in the immediate post-transplantation period. [48]. L FA-1 is expressed on all hematopoietic cells including memory T cells and NK cells. Thus, blocking LFA-1 may prolong graft survival even in sensitized patients. However, we show here that even triple costimulation blockade did not prolong graft survival in sensitized recipients.

Several studies have examined the effect of additional costimulation blockade combinations on graft survival in sensitized recipients. Some reagents such as anti-OX40L Abs have shown promising effects in rodents [74].

Culture *in vitro* of T cells with donor-specific antigens and co-stimulatory blockade facilitates generation of donor-specific regulatory T cells (Treg) [75]. Clinical trials using such *in vitro* generated Treg cells for graft-specific immunosuppression have been initiated.

Costimulation blockade is useful to induce energy or tolerance to antigens. However, additions to this therapy may be needed. We have recently observed that a combination of co-stimulatory blockade and mesenchymal stem cells improved islet graft survival in a rodent model compared to costimulation blockade or MSCs alone.

4.2 PAPER II

4.2.1 Early effects of immune antibodies on islet xenografts

The early effect of immune antibodies (Fig. 4A) on xenografts was examined at 48 and 96 hours after transplantation. Recipient mice were transplanted with rat islets, injected intraperitoneal with naïve or immune serum and treated with control Abs or triple (anti-CD154, CTLA4Ig and anti-LFA-1) costimulation blockade. At both 48 and 96 hours post-rat islet transplantation/post-serum injection, anti-rat splenocyte IgG Abs were detected in mice injected i.p. with immune, but not naïve, serum (Fig. 4B and C).

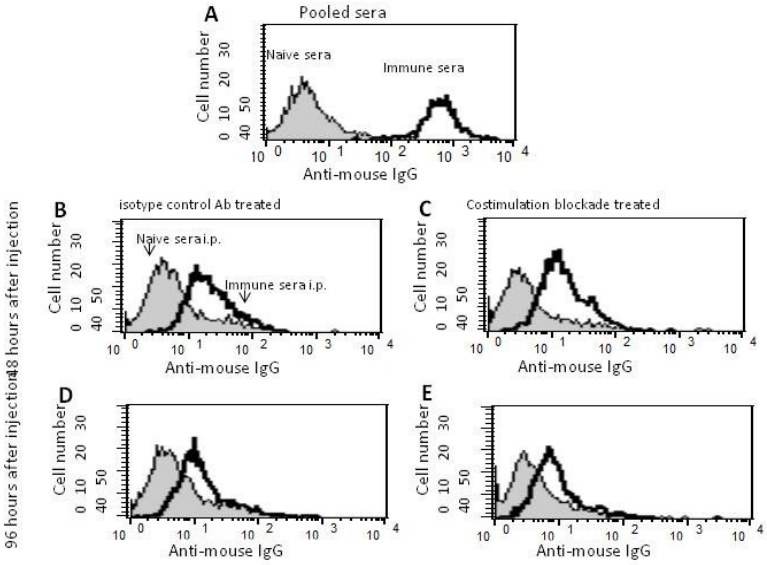


Figure 4 Detection of anti-Lewis rat antibodies in mouse serum samples using flow cytometry
Anti-rat antibodies (Abs) in pooled serum from sensitized (n=17; thick black line) and naïve (n=5; filled curve) C57BL/6 mice were analyzed by flow cytometry using rat splenocytes as target cells (A). The serum samples shown in A were used as immune and naïve serum in the mice analyzed in B-G. The serum of mice transplanted with rat islets, injected with naïve (filled curve) or immune (thick black line) serum, and receiving control Abs (B and D) or costimulation blockade (C and E) were analyzed 48 (B and C) or 96 (D and E) hours post-transplantation (post-Tx) using flow cytometry and rat splenocytes as target cells.

The average MCF of mouse anti-rat IgG Abs detected by flow cytometry in mice of each group is shown in Tables 3A and B. At 48 hours after transplantation, immune serum-injected

groups showed significantly higher levels of anti-rat Abs than naïve serum-injected groups. At 96 hours the difference was less pronounced (Fig. 4E and F).

The effect of immune serum on rat islet xenograft morphology 48 and 96 hours post-Tx in recipients receiving costimulation blockade or control Abs was evaluated by histological examination of graft sections. At 48 hours, the graft mass and the number and type of infiltrating cells were similar in the groups of mice injected with naïve and immune serum, respectively (Table 3A). Even though statistical significance was not reached, there appeared to be slightly more TUNEL-positive (apoptotic) cells in the group given immune serum and costimulation blockade compared to the group receiving naïve serum and costimulation blockade (Table 3A, Fig. 5A). Histology sections did not show significant differences between the groups. PMN and MNC were seen 96 hours post-transplantation in the groups treated with control Abs irrespective of whether they were given naïve or immune serum (Table 3A and B).

Table 3A: Histological scoring of grafts recovered 48 hours post-transplantation

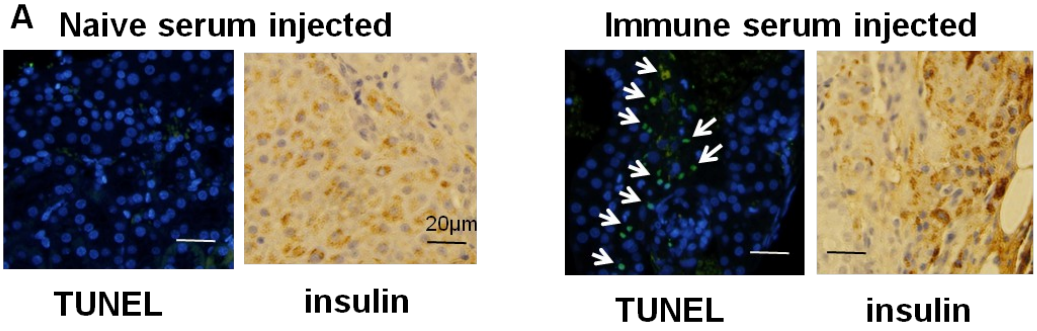
	n	anti-rat [a]Abs [b]MCF	[d]Graft mass	[d]Infiltrating cells MNC	PMN	[d]Apoptotic cells
naïve serum, isotype control	3	9.9±3.1	1.6±0.3	1.2±0.7	1.1±0.5	1.3±0.3
immune serum, isotype control	4	[c]26.0±5.5	1.4±0.6	1.3±0.3	1.4±0.3	1.6±0.3
naïve serum, triple Ab	4	8.6±1.7	1.8±0.9	1.4±0.5	1.6±0.5	1.0±0.7
immune serum, triple Ab	4	[c]25.9±2.9	1.8±0.5	0.7±0.5	0.8±0.8	2.1±0.9

Table 3B: Histological scoring of grafts recovered 96 hours post-transplantation

	n	anti-rat [a]Abs [b]MCF	[d]Graft mass	[d]Infiltrating cells MNC	PMN	[d]Apoptotic cells
naive serum, isotype control	3	13.1±5.0	1.6±0.5	2	1.4±0.5	1.3±0.6
immune serum, isotype control	4	[e]21.0±3.7	1.5±0.7	1.7±0.5	1.2±0.4	1.2±0.4
naïve serum, triple Ab	3	9.0±1.0	3	0.1±0.1	0.1±0.1	1.3±0.6
immune serum, triple Ab	3	14.0±1.3	3	0.8±0.2	0.1±0.1	0.7±0.6

[a]Abs, antibodies
[b]MCF, mean channel fluorescence
[c]$P<0.01$ compared with naïve sera injected groups (Table 3A)
[e]$P<0.05$ compared with naïve sera injected groups (Table 3B)
[d]Each graft was scored by two persons and the Table shows the mean score±SD of each group. For definitions of the score, see the Materials and Methods

A


Naive serum injected
Immune serum injected
20μm
TUNEL
insulin
TUNEL
insulin

B

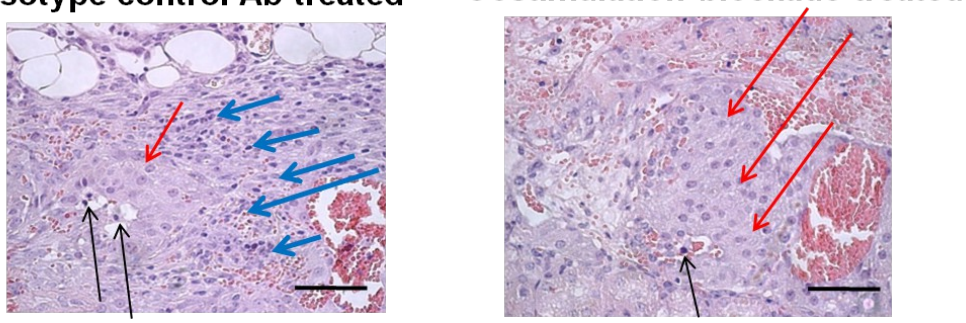

Isotype control Ab treated
Costimulation blockade treated

Figure 5 Histological examination of early effect of immune antibodies on the xenografts.
Mouse kidneys bearing rat islet xenografts were explanted at 48 and 96 hours after transplantation, sectioned and analyzed with regard to TUNEL (white arrows) and insulin (brown) positive cells (A; 48 hours). Bars, 20 mm. The effect of the costimulation blockade was seen at 96 hours post-Tx. Grafts from mice given isotype control antibodies (B left) exhibited a moderate infiltration of polymorphonuclear and mononuclear cells (blue arrows), while grafts receiving triple costimulation blockade (B right) had fewer infiltrating cells and well-maintained endocrine tissue (red arrows). Only a few TUNEL-positive cells were observed in grafts harvested at 96 hours post-Tx (black arrows). Magnification: 40x and bars, 50 mm.

4.2.2 Rat islet xenograft survival in mice treated with costimulation blockade

C57BL/6 mice were divided into six groups (Table 1), control (groups 1 and 2), double (groups 3 and 4) and triple (groups 5 and 6) costimulation groups given naïve and immune serum, respectively. Blood glucose and body weight were followed in all animals. Control groups rejected their grafts within two weeks, while mice in groups receiving costimulation blockade (groups 3-6) had prolonged graft survival. No statistical differences were seen between immune and naïve serum-injected groups, *i.e.* group 1 vs. 2, group 3 vs. 4 and group 5 vs. 6 (Fig. 6). IPGTT was performed one and four months after transplantation in order to further examine graft function. Following the intraperitoneal glucose challenge, blood glucose recovery was similar between naïve and immune serum-injected groups.

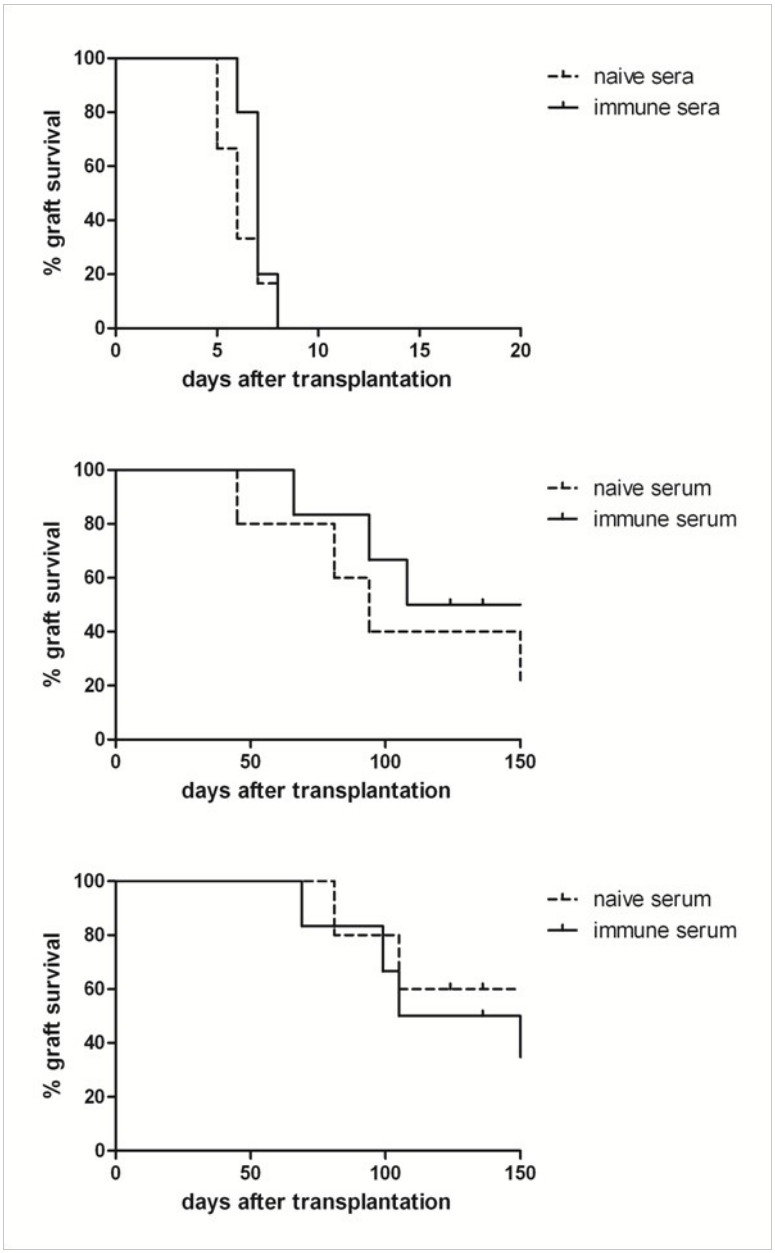


Figure 6 No effect of donor-specific antibodies on islet xenograft survival prolonged by costimulation blockade Rat islet xenograft survival (%) in mice receiving isotype control antibodies (A), double (B) or triple (C) costimulation blockade. Half of the mice in each group received naïve (broken lines) and half immune (solid lines) mouse serum. Rejection was defined based on the non-fasting blood glucose levels as described in Materials and Methods.

Explanted grafts were further assessed by histopathological examination. Hematoxylin and eosin staining, anti-insulin and TUNEL staining were performed. Sections from group 1 and 2 showed many apoptotic cells and very few insulin positive cells, infiltration of MN and PMN cells were seen. In the functional grafts, insulin positive cells were clear and no/very few apoptotic cells were found, while the rejected grafts had massive MNC infiltration and many TUNEL positive cells. A few mice in groups 5 and 6 had functional grafts but many TUNEL+ cells, which may suggest early stage graft rejection (Table 4; Fig. 7 and 8).

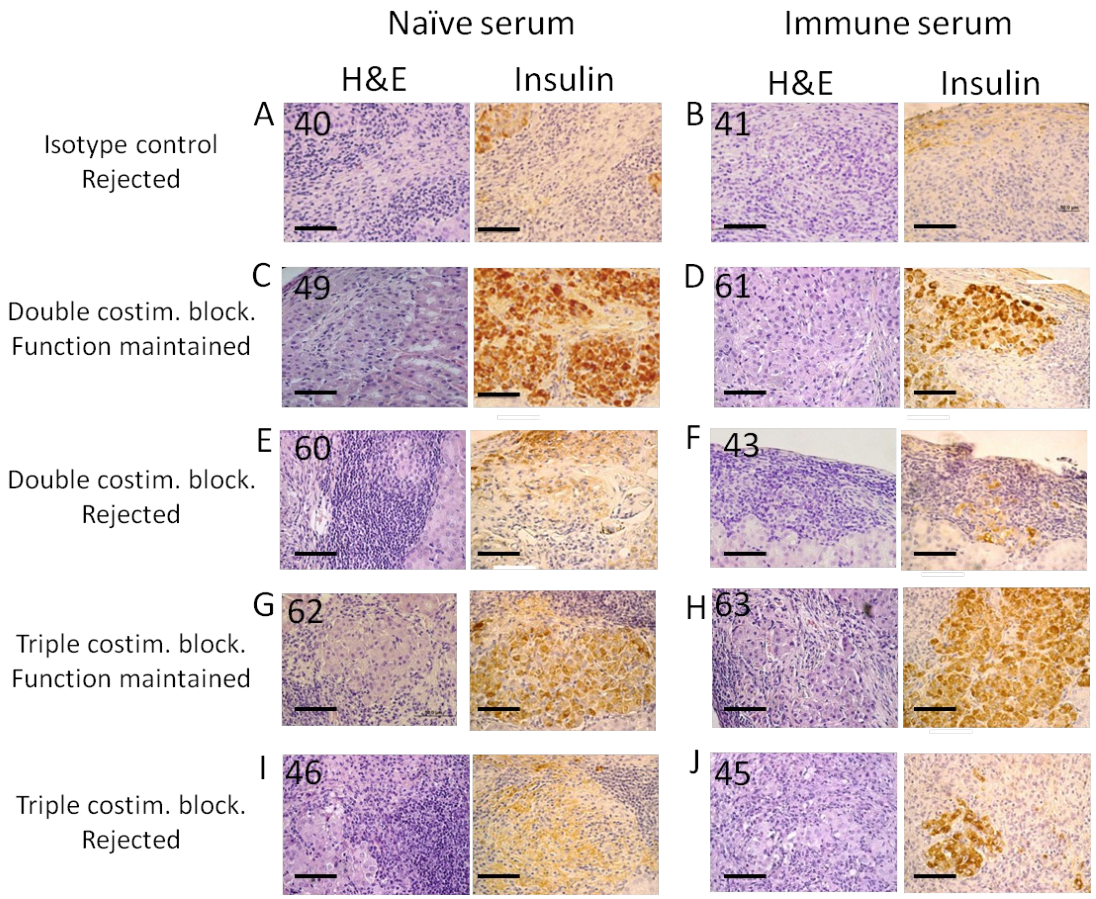


Figure 7 Sections of explanted rat islet grafts from mice treated by costimulation blockade and given naïve or immune mouse serum were stained by hematoxylin & eosin and anti-insulin antibodies.
Explanted grafts from mice in each group were sectioned and stained by hematoxylin and eosin (H&E) and anti-insulin antibodies. Insulin-positive cells are stained brown. A representative section from one graft in each group is shown in panels A to J. The numbers on the left upper corner of each picture correspond to the mouse ID number referred to in Table 4. The sections were from a graft out of the group receiving isotype control antibodies (Abs) and naïve serum (A), isotype control Abs and immune serum (B), a functional graft from the group treated with double costimulation blockade and given naïve serum (C), and a rejected graft from the same group (E). In panel D a section of a functional graft from the group receiving double costimulation blockade and immune serum is shown, and in F is shown a section of a rejected graft from the same group. A functional graft from the group receiving triple costimulation blockade and naïve serum is shown in G and a section from a rejected graft of the same group is shown in panel I, while in H a functional graft from the group treated by triple costimulation blockade and given an intraperitoneal injection of immune serum is shown and in panel J we show a rejected graft from the same group. The original magnification was 40x and bars indicate 50 mm.

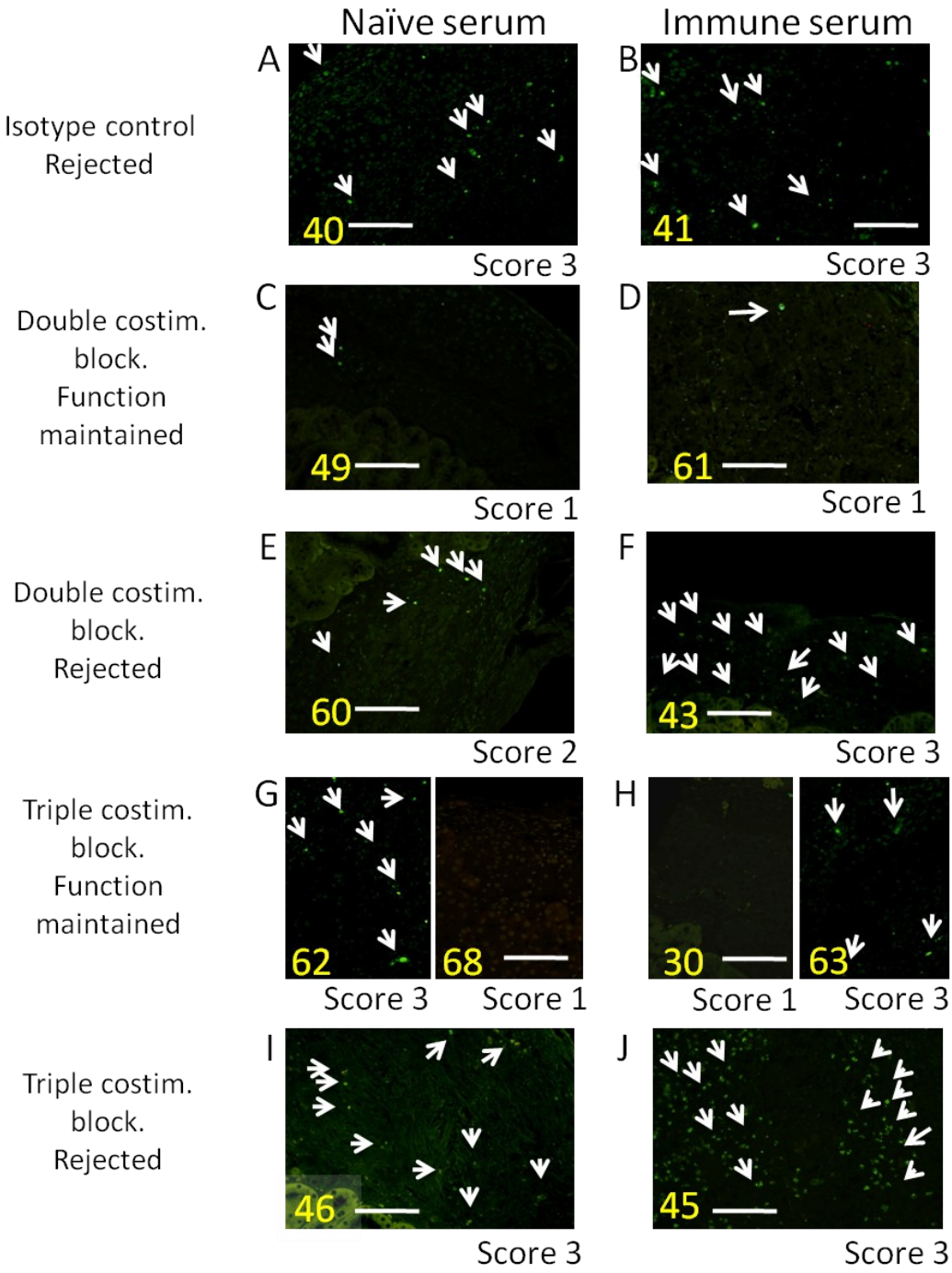


Figure 8 Detection of apoptotic cells using the TUNEL assay on explanted islet grafts.
Explanted grafts from mice in each study group were also examined by the TUNEL assay in order to quantify the number of apoptotic cells. The sections are from the same recipients and presented in the same order as shown in Fig. 6. Green spots represent TUNEL positive cells. White arrows point at TUNEL positive cells. The scores given under each panel can be found in Table 4 and how they were calculated is described in the Materials and Methods section. The bars indicate 100 mm.

Explanation of Table 4 below:
ND: not done because of technical failure
aGraft function at the time of graftectomy. Maintained: a non-fasting blood glucose level of <10 mM; Rejected: blood glucose levels in two consecutive samples >20 mM.
bScore shows mean ±SD from three different levels of samples. When all the scores were the same, SD (±0) was left out.
cNo TUNEL staining, number of apoptotic cells estimated from HE stained slides

Table 4: Transplantation results, anti-donor antibodies and histology scoring in each group

			Histological analysis (score)[b]			
				Infiltrating cells		
Mouse ID	Graft function[a]	Anti-rat antibodies(IgG; MCF)	Endocrine tissue	MNC	PMN	Apoptotic cells
Group 1						
#7	Rejected	51.57	1.3±0.57	2	2.7±0.57	3
#26	Rejected	38.85	1	2	1	3
#40	Rejected	34.84	1	2	2.7±0.57	3
#51	Rejected	ND	0	3	1	3
Group 2						
#27	Rejected	43.74	0.3±0.57	2.7±0.57	1.7±0.57	ND
#41	Rejected	49.99	1	2.3±0.57	1.7±0.57	3
#50	Rejected	71.79	1.3±0.57	2.7±0.57	2.3±0.57	3
#52	Rejected	27.12	0.3±0.57	2.3±0.57	1	3
#59	Rejected	194.78	0.3±0.57	1.7±0.57	1.7±0.57	3
Group 3						
#49	Maintained	13.17	3	0	0	1
#28	Rejected	14.10	1.3±0.57	1.7±0.57	1.3±0.57	2
#42	Rejected	22.99	2.3±0.57	2±1	1	3[c]
#55	Rejected	6.95	2.7±0.57	2.7±0.57	1	1,3±0,55
#60	Rejected	6.64	1.7±0.57	0.7±1.15	2	2
Group 4						
#38	Maintained	10.25	2.3±1.2	0	0	0
#61	Maintained	3.10	2.7±0.57	0.7±0.57	0	1
#67	Maintained	16.98	ND	ND	ND	ND
#39	Rejected	97.82	0.7±0.57	3	1.7±0.57	3
#43	Rejected	7.04	0.7±0.57	2.3±0.57	1.3±0.57	1
#48	Rejected	254.07	0.3±0.57	3	1.7±0.57	3
Group 5						
#62	Maintained	69.28	2	2.3±1.15	1.3±0.57	3
#68	Maintained	16.63	3	1	0.7±0.57	1
#46	Rejected	93.19	0	3	1.7±0.57	3
#47	Rejected	28.54	0.7±0.57	3	1.7±0.57	3
#57	Rejected	ND	1.3±0.57	2.3±1.15	0.7±0.57	3
Group 6						
#30	Maintained	73.49	2.3±0.57	2.3±1.15	0.7±0.57	1
#63	Maintained	79.91	3	1.7±0.57	0.7±0.57	3
#12	Rejected	61.71	0.7±0.57	3	2	3
#45	Rejected	221.67	1.3±0.57	2.7±0.57	2	3
#56	Rejected	367.45	3	3	1.3±0.57	3**
#69	Rejected	ND	0	3	2.3±1.15	3

Taken together, our study indicates that the existence of donor-specific antibodies alone at the time of transplantation do not affect graft survival irrespective of whether the mice received costimulation blockade or not.

4.2.3 Discussion

Previous studies have investigated the effect of antibodies, either adoptively transferred or induced in the recipient prior to transplantation by injection of donor cells/antigens, in discordant pig-to-rodent models. Hyper-immune antibodies were often generated by repeated injections of splenocytes from the donor species and included both anti-MHC class I and II antibodies of both IgM and IgG class. In general, immune serum injections in recipient rats or mice accelerated xenograft rejection [76-78].

In our study, we did not observe any effect of immune antibodies on graft survival. We immunized mice by islet transplantation in order to get islet graft-specific antibodies. No second challenge with antigen was performed. The transplanted islet grafts were normally cultured for 20 hours, and during culture, some infiltrating macrophages and exocrine tissue may disappear, which makes islets less immunogenic. IgG antibody titers increased between week 3 and 4, and were maintained up to 6-7 weeks post-transplantation, even though the grafts were normally rejected around POD 7. The immune serum samples were collected 4-6 weeks after transplantation. At that time, IgM antibodies may not be present, even though we detected both IgM and IgG antibodies in pooled immune serum. In paper I, we used a similar method for sensitization, which was enough to prevent long-term graft survival by costimulation blockade. Thus, in our model anti-donor antibody did not affect graft survival or the ability of costimulation blockade to induce long-term graft survival. Taken together the results of paper I and II suggests that in order to achieve long-term graft survival in sensitized recipients one should focus on preventing memory T activation. A potential point of attack, besides the memory T-cells themselves, to accomplish this could be to control also the dendritic cell population, which is known to influence the development of Treg or memory T cells.

4.3 Paper III

4.3.1 No clear effect of ARA 290 on rat islets during culture

To evaluate effect of ARA 290 on the islet culture, we added various concentrations of ARA 290 to the culture medium and the cells were cultured for 48 hours. Caspase activity increased during the 48 hours of culturing, most likely due to hypoxia and central necrosis in the islet core. No significant difference was observed among any of the treatment doses.

4.3.2 ARA 290 protects islets from pro-inflammatory cytokine-mediated cytotoxicity

To mimic the inflammatory situation, a mixture of proinflammatory cytokines was added in islet culture medium with/without ARA 290 (100 nM). Other study indicated 10 nM ARA 290 is sufficient to protect tissue damages (personal communication with Professor Östenson Karolinska Institutet, Sweden and professor Cerami, Leiden University Medical Center, Leiden, the Netherlands). In our hand, ARA 100 nM in culture did not show any toxic effect on rat islets. Therefore, we decide to use 100 nM ARA 290 as coculture concentration with proinflammatory cytokines.

4.3.2.1 Caspase 3/7 activities

The caspase 3/7 activity of islets stimulated with pro-inflammatory cytokines for six hours was significantly lower in the ARA 290 treated group than in the cytokine group (cytokine and ARA treated group: 152±18 vs. cytokine group: 182±18 luminescence/dsDNA(ng), $p<0.05$). During 12 hours of incubation, the ratio increased but the ARA 290 treated group still showed a lower value than the cytokine group (199±25 vs. 172±21, respectively *n.s.*) (Figure 9).

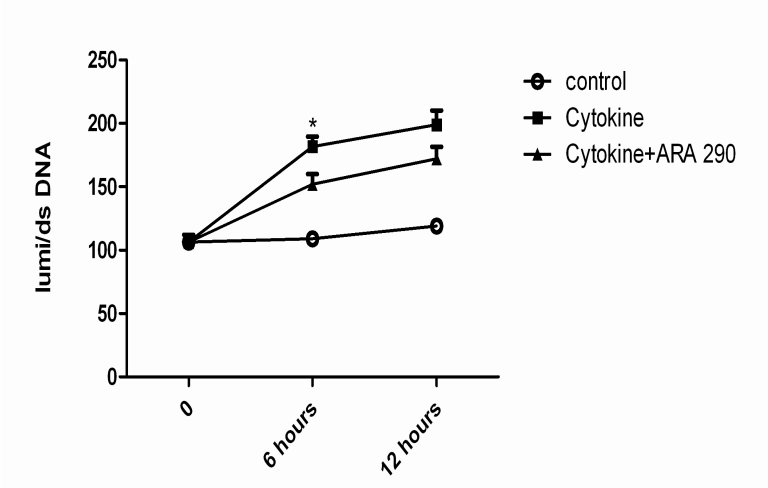
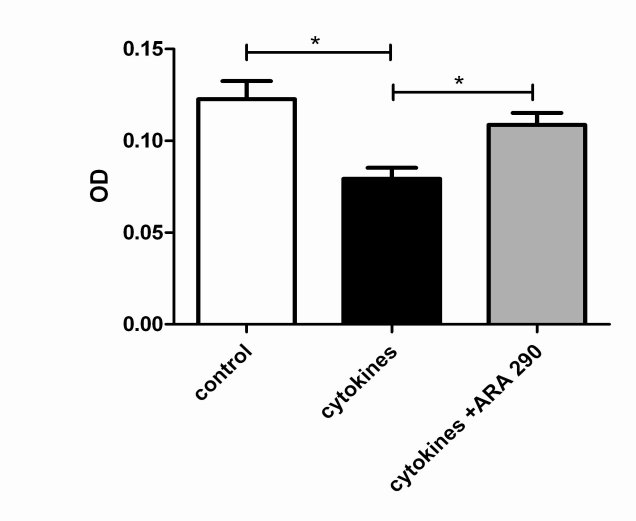


Figure 9 Caspace 3/7 activities of islets stimulated with proinflammatory cytokines.
50 islets were cultures in 35 mm plate and stimulated with proinflammatory cytokines with or without ARA290 treatment. After 6 hours and 12 hours, Caspace 3/7 activities were measured and islet amount was corrected by dsDNA (ng/ml).

4.3.2.2 Islet viability (MTT assay)

In order to assess viability of islets, the MTT assay was performed with islets exposed to pro-inflammatory cytokines with/without ARA 290 for 6 hours. The viabilities of the islets were measured as OD values (Figure 10). The viability of the islets stimulated by cytokines for 6 hours was clearly reduced but the ARA 290 treated group maintained the viability

significantly better (the % values of non-cytokine exposed islets was 55±4.5 vs. 75±5.6 %,



respectively, $p<0.05$)

Figure 10 Viabilities of islets stimulated with proinflammatory cytokines, assessed by the MTT assay.
MTT assays were performed with 20 islets per well. The islets were incubated with proinflammatory cytokines with or without addition of ARA 290 for 6 hours. The figure shown is % OD values compared with islets without cytokine activation.

4.3.2.3 ARA 290 preserved beta cell function in the presence of pro-inflammatory cytokines

The capacity of islets to secrete insulin in response to high concentrations of glucose was tested in SGIS. In responses to low glucose, no significant differences were observed regardless of the presence of ARA 290. In the responses to high glucose, the islets in the cytokine and ARA 290-treated group secreted significantly higher insulin than those in the cytokine alone group (0.74±0.10 vs. 0.33±0.03 ng/ml, respectively) (Figure 11A). In addition, the stimulation index in the cytokine and ARA 290-treated group was also higher than that in the cytokine alone group (1.67±0.22 vs. 0.97±0.15, respectively) (Figure 11B).

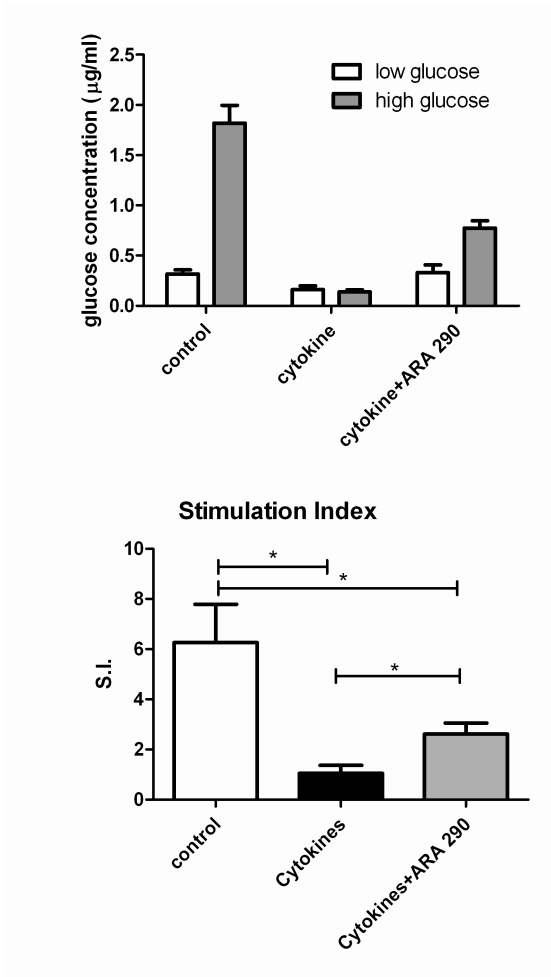

glucose concentration (µg/ml)
2.5
2.0
1.5
1.0
0.5
0.0
low glucose
high glucose
control
cytokine
cytokine+ARA 290
Stimulation Index
S.I.
10
8
6
4
2
0
*
*
*
control
Cytokines
Cytokines+ARA 290

Figure 11 Static glucose-stimulated insulin secretion (SGIS) of islets after stimulation by proinflammatory cytokines with or without ARA 290. The islets (20/well) were incubated for 6 hours with proinflammatory cytokines (Cytokine) or proinflammatory cytokines with ARA290 (Cytokine+ARA 290). Shown are: (A) the insulin concentration in response to low glucose, (open bars) and high glucose (filled bars) and the (B) stimulation index (insulin concentration in high glucose/insulin concentration in low glucose) of each group. *$p<0.05$ insulin amount at high glocuse stimulation in Cytokine group vs. in Cytokine+ARA 290 group. Mean ± SD of 4 experiments are shown.

Taken together, ARA 290 could enhance the resistance of the islet cells against cytokine-induced apoptosis.

4.3.3 The effect of ARA 290 treatment on islet graft function during syngeneic islets transplantation of a marginal mass

4.3.3.1 Non-fasting blood glucose and body weight

The effect of ARA 290 treatment was examined in rat syngeneic islet transplantation. In this series, recipients were transplanted with 220 islets and ARA 290 injections were administered subcutaneously for two weeks. In the control group, two out of five rats showed partial function of the engrafted islets during the fourth week after transplantation (blood glucose between 10mM and 20 mM) and in the ARA 290 treated group, one out of four rats (25%) was cured after 14 post-operative days (POD). No significant differences were observed in the non-fasting blood glucose levels between the two groups during 28 days of observation.

To further characterize the graft function, OGTT was performed in the fourth week after transplantation (2-3 days before graftectomy). Transplanted groups showed delayed recovery of blood glucose levels compared with naïve control, however, there was no differences between the two transplanted groups.

The insulin amount of the grafts was also examined four weeks after transplantation. In the mice that were not cured, almost no insulin was detected in the graft bearing kidney, whereas the cured mice showed 3-7 μg of insulin. The results are summarized in Table 5.

Table 5: Results of marginal mass islet transplantation study

	Control Group	ARA 290 treated group	p-value
number of animals	5	4	
failure	3	3	
partial cure	2	0	
cure	0	1	
% cure	0%	25%	ns
mean of non-fasting blood glucose (mM)	23.8±5.4	20.9±6.4	ns
graft insulin (μg)	1.2 (range 0.1-3.0)	1.9 (0.1-7.4)	ns
body weight gain (g)	20±11	21±8	ns
OGTT: AUC in blood glucose mMx120	2365±703	1286±593	ns

min			
OGTT: AUC in plasma insulin µg/mL x60 min	23,0±10.4	23.8±6,2	ns

4.3.3.2 OGTT

To further characterize the graft function, OGTT was performed in the fourth week after transplantation (2-3 days before graftectomy). Transplanted groups showed delayed recovery of blood glucose levels compared with naïve controls; however, there were no differences between the two transplanted groups.

The insulin amount of the grafts was also examined four weeks after transplantation. In the mice that were not cured, almost no insulin was detected in the graft bearing kidney, whereas the cured mice showed 3-7 µg of insulin.

4.3.4 Discussion

The mechanism by which EPO protects tissues from damage *in vivo* is not fully understood. The expression of the EPO receptor on non-hematopoietic cells is not very high. It is upregulated by inflammation. Therefore, its effects became more evident when target cells were incubated with proinflammatory cytokines [58]. Islets in culture, where inflammatory mediators are sparse, did not respond to ARA 290, perhaps because of lack of receptor.

We have also tested the effect of ARA 290 during islet isolation with or without cold ischemia. In contrast to the clinical situation when islets are derived from brain dead, deceased donors, the exposure of islets from animal donors for pro-inflammatory cytokines can be anticipated to be very limited. Donor rat treatment with ARA 290 or co-injection of ARA 290 with collagenase solution did not improve islet yields, viability or function. The results were similar in an islet isolation model with prolonged cold ischemia (18 hours).

The effect of ARA 290 on graft survival after transplantation was also examined using an under the kidney capsule transplantation model. This model does not cause very severe inflammation compared to islet transplantation via portal vein injection. Two different treatment protocols were tested in the syngeneic rat transplantation model under kidney capsule, but no clear differences were observed with or without ARA 290. Protective effects of ARA 290 on islets following exposure to pro-inflammatory cytokines have been repeatedly observed in both mice and rat islets. In the type 2 diabetes model, Goto-Kakizaki rats, ARA 290 protected the islets from apoptosis and increased their glucose sensitivity. [79]

Therefore, we will further evaluate the effect of ARA 290 on islet graft function after islet transplantation via the portal vein.

One key component in order to achieve prolonged islet graft function is to regulate macrophage activity. They are activated during islet transplantation via the portal vein and activated macrophages produce free radicals that impair islet function and reduce islet viability. NF$^{\varkappa}$ B is a key factor for activation of macrophages. The reports regarding the effect of EPO on NF$^{\varkappa}$ B regulation and thereby macrophage activity are controversial. [80] If ARA 290 can down regulate macrophage activity, it could become a useful treatment as an anti-inflammatory drug.

4.4 PAPER IV

4.4.1 Mice injected with STZ lost more body weight than mice injected with alloxan

The general effects of STZ and ALX on mice were assessed by measuring their body weight and blood glucose levels following injection. All mice showed elevated blood glucose levels 48 hours after drug injection. During the four day observation period, mice with ALX-induced diabetes had significantly higher blood glucose levels at day two compared to mice injected with STZ (33.2±2.3 vs. 19.7±5.6; $p<0.001$; Figure 12A). STZ-injected mice appeared weaker, as indicated by less movement, and lost more body weight than ALX-injected mice [11.5±1.8% (n=7) vs. 7.13±0.8 (n=15), p=0.049; Figure 12B].

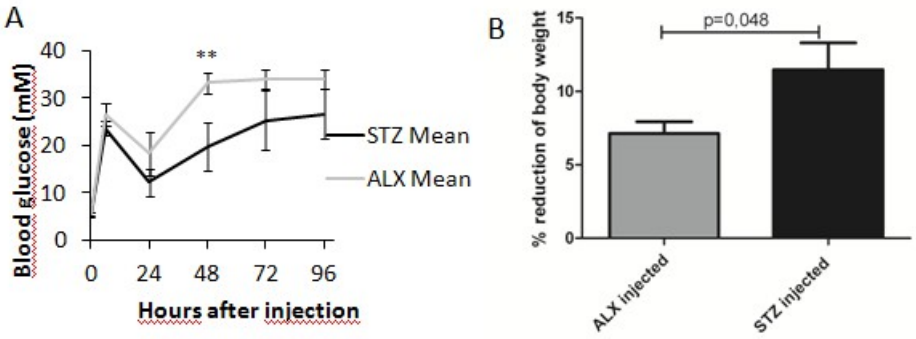


Figure 12 Changes of blood glucose and body weight after STZ or ALX injection
Blood glucose and body weight during four days after ALX or STZ injection was monitored. (A) Mean ±SD of non-fasting blood glucose of ALX iv group (n=15, grey line) and STZ iv group (n=7, black line) is shown. Blood glucose was measured before injection, 6 hours after injection and days 1, 2, 3 and 4 after injection. (B) % reduction of body weight during four days was calculated and compared.

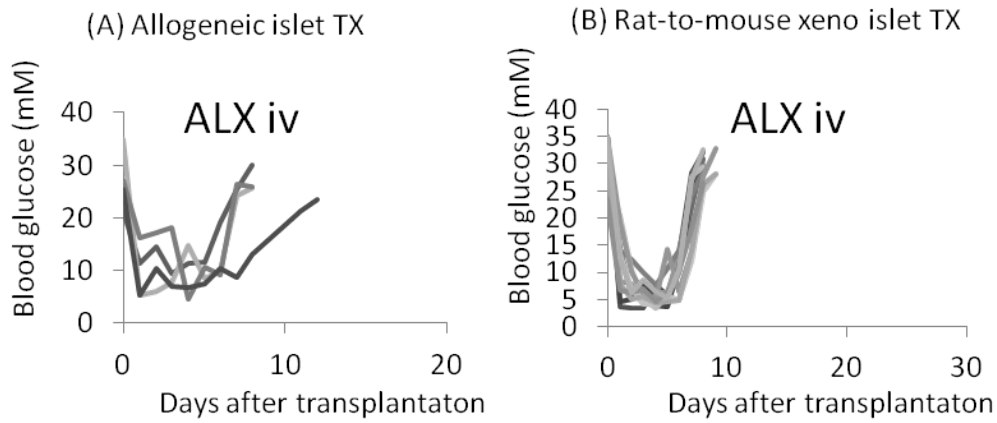
4.4.2 STZ injected mice had fewer splenocytes than ALX-injected mice

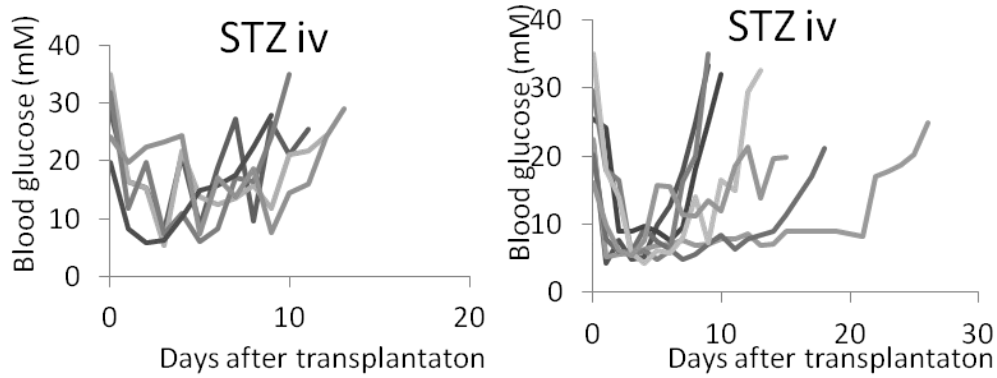
Spleens were harvested at four days after drug injection and recovered splenocytes were counted. STZ-injected mice had significantly fewer splenocytes (9.2±3.2 x 10^6; n=3)

compared to mice injected with ALX (22.7±9.3 x 10^6; n=3; $p<0.05$). Both ALX- and STZ-injected mice had significantly fewer splenocytes than healthy control mice (65.7±3.5 x 10^6; n=3; $p<0.05$).

4.4.3 Islet xenograft survival was longer in STZ-injected than in ALX-injected mice

Graft survival was monitored by daily measurements of non-fasting blood glucose. The day of graft rejection was set to be the first post-operative day that recipient mice had non-fasting blood glucose levels over 20 mM for more than two consecutive days. Allogeneic islets survived for 6-10 days in the ALX-injected group (n=4) and for 7-11 days in the STZ-injected group (n=5, ns: between the groups) (Figure 13). Xenogeneic rat islets survived for 6-7 days in the ALX-treated group (n=11) and 7-24 days in the STZ-treated group (n=7; p=0.0006, Figure 13).


(A) Allogeneic islet TX
(B) Rat-to-mouse xeno islet TX
ALX iv
ALX iv
Blood glucose (mM)
Days after transplantaton


STZ iv
STZ iv
Blood glucose (mM)
Days after transplantaton

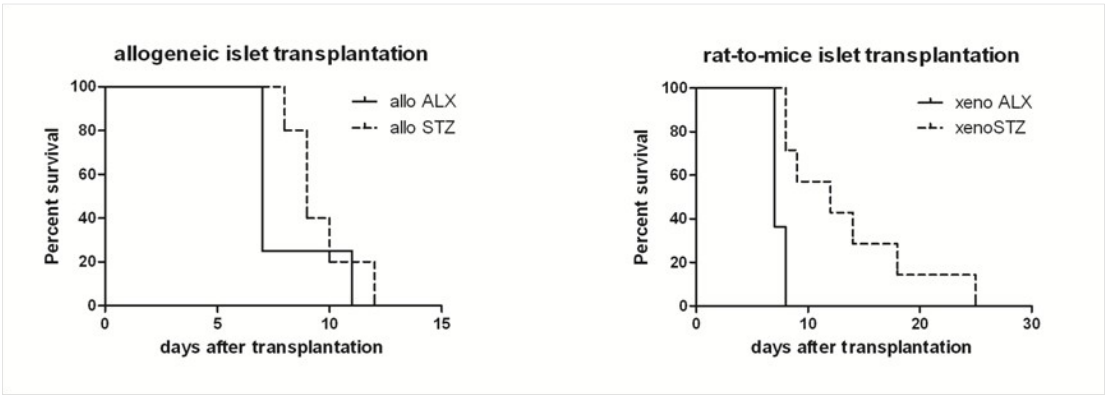

allogeneic islet transplantation
rat-to-mice islet transplantation
allo ALX
allo STZ
xeno ALX
xenoSTZ
Percent survival
days after transplantation

Figure 13 Non-fasting blood glucose after islet transplantation
Diabetes was induced in C57BL/6 mice by an iv injection of STZ (180 mg/kg) or ALX (75 mg/kg). Panel (A) shows non-fasting blood glucose after allogeneic islet transplantation (upper panel ALX iv, n=4, middle panel STZ iv, n=5) and survival curve (lower panel). Panel (B) shows non-fasting blood glucose levels after rat-to-mouse xenogeneic islet transplantation (ALX iv, n=11, and STZ iv, n=7) and survival curve. Time to rejection of transplanted grafts was longer in mice injected iv with STZ compared to that seen in ALX injected mice. The difference was significant (p=0.0006) in the xenotransplantation model.

4.4.4 STZ and ALX cytotoxicity on human and murine leukemic cells

ALX appeared not to be toxic for the studied cell lines with estimated IC_{50} values of 2809, 3679 or over 4000µg/ml for HL60, K562 and C1498 cells, respectively. STZ was more toxic, especially for the human myeloid leukemia cell line, HL60. The IC_{50} values of STZ were 11.7, 904 and 1024 µg/ml for HL60, K562 and C1498 cells, respectively. Results also showed that the murine leukemic cells are more resistant to STZ and ALX cytotoxicity than human leukemic cells (Table 6)

Drug name	**HL-60 cells** IC50 (µg/mL)	**K562 cells** IC50 (µg/mL)	**C1498 cells** IC50 (µg/mL)
Streptozotocin	11.7	904	1024
Alloxan	2809	3679	>4000

Table 6: Fifty percent inhibitory concentrations (IC50) of Streptozotocin and Alloxan on human (K562 and HL-60) and murine (C1498) leukemia cell lines.

5 CONCLUSIONS

Triple costimulation blockade with a combination of CTLA4Ig, anti-CD154 and anti-LFA-1 antibodies were not sufficient to prolong allogeneic islet graft survival in sensitized recipients.

Immune serum did not affect rat islet xenografts in the immediate post-transplantation period or the graft survival long-term. The treatment efficiency of costimulation blockade was preserved despite administration of immune serum.

The erythropoietin analogue, ARA 290, which is lack of hematopoietic function but tissue protective, may protect also islets from damage caused by inflammation.

Streptozotocin appeared more toxic on immune cells *in vivo* and more cytotoxic on leukemia cells *in vitro* than alloxan. Thus, ALX is a better reagent for diabetes induction in islet transplantation models.

6 FUTURE WORK

Based on the work in this thesis there are many outstanding questions to resolve in order to make islet transplantation the treatment modality of choice for patients with diabetes:

Improving islet quality: Improved islet isolation procedures that preserves islet viability and functionality are needed. Addition of agents with a cytoprotective effect could be one way of accomplishing this. We will further evaluate ARA 290 in that respect.

Improving islet engraftment. Important for the future will be to prevent IBMIR and to promote engraftment. Understanding the immediate events following intraportal injection of islets, and preventing those will be essential in order to be able to decrease the number of donors needed for each patient. Gene therapeutic approaches by which factors are expressed in the graft that can interfere with IBMIR may be a path forward to improve the number of surviving islets.

Preventing graft rejection: Further studies are needed to delineate the effect of different blockers of costimulation, particularly in terms of inhibiting memory T-cells. Additional blocking agents including inhibitors of the integrins VLA-4 (CD49d/CD29) and LFA-1 (CD11a/CD18), the CD134:CD134L pathway, the IL-2 receptor β chain, CD122, or the CD2:LFA-3 (CD58) interaction should be investigated in models with sensitized recipients.

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This list is absolutely non-exhaustive.

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