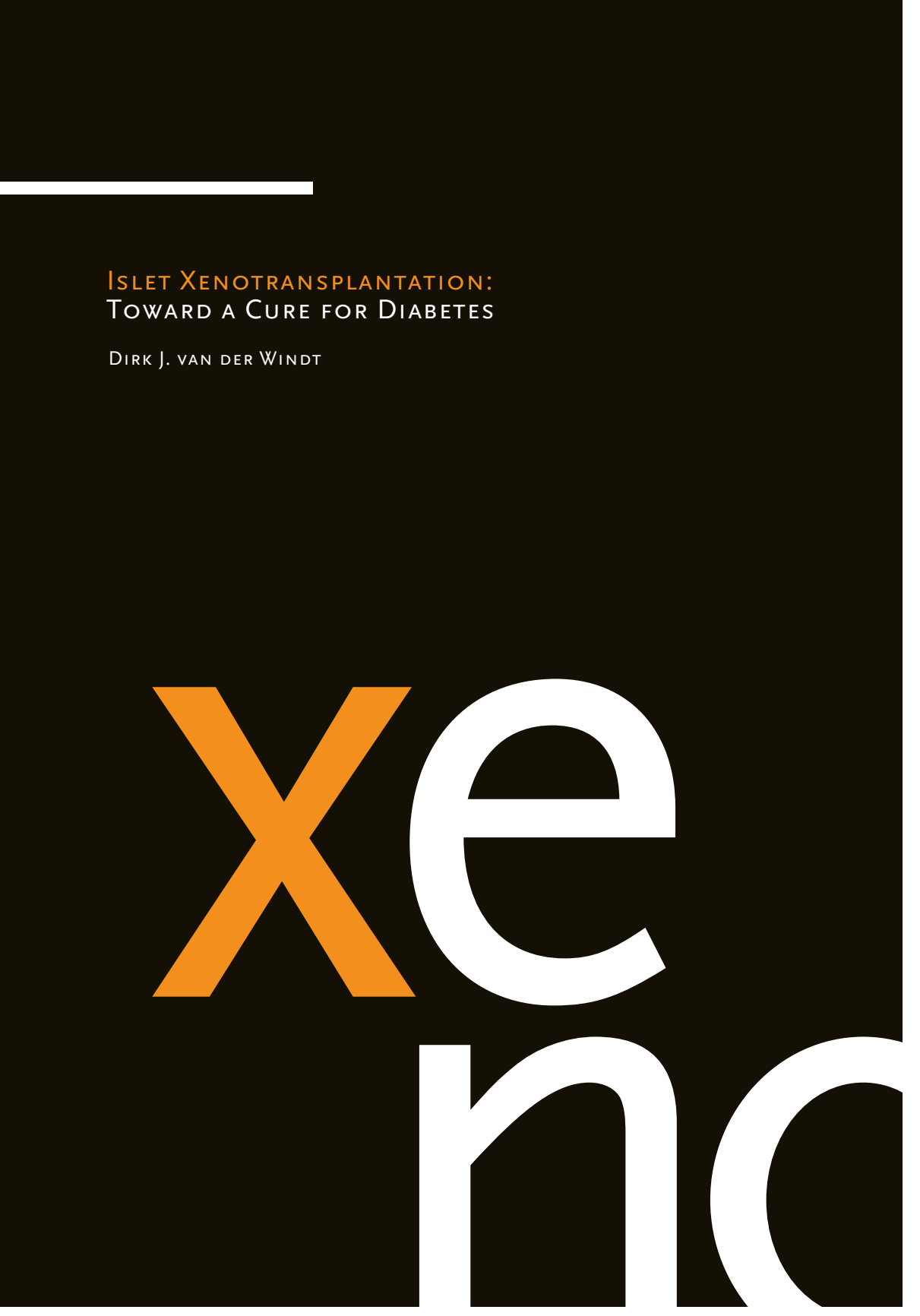
Islet Xenotransplantation: Toward a Cure for Diabetes

Dirk J. van der Windt



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de weg naar genezing van diabetes

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Voor mijn ouders

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INTRODUCTION

CHAPTER 1

General introduction & aims

General Introduction

TYPE 1 DIABETES

The pancreas is an organ with a central anatomical position in the abdomen, and a central role in the processes of food digestion and glucose metabolism. The vast majority of pancreatic tissue (more than 98%) is dedicated to the production and secretion of digestive enzymes that are released into the proximal small intestine, and therefore has an exocrine function. The remaining 1-2% is composed of cells producing mainly insulin (by beta cells) and glucagon (by alpha cells), hormones that are released into the bloodstream to regulate blood glucose levels. This small part of the pancreas therefore has an endocrine function. Those hormone-producing cells are organized in small clusters that appear as 'small islands' (islets) in the 'sea' of exocrine tissue (Figure 1), as first described by a German pathologist, Paul Langerhans, in 1869 while he was still a medical student, although at that time he was unaware of their function. When later their crucial role in glucose metabolism was discovered, they became known as islets of Langerhans, in the fields of diabetes and transplantation (Tx) simply referred to as 'islets'.

If insulin production by islets fails, which is the case in type 1 diabetes (T1D), blood glucose levels rise. In this situation, the amount of glucose filtered by the kidney will exceed the kidney's capacity to reabsorb glucose, and glucose will be found in the patient's urine ('diabetes mellitus' literally means 'excessively sweet urine'). Without insulin, the glucose molecules circulating in the bloodstream cannot enter tissues such as the liver and muscles, where they are needed as fuel. Instead, those tissues will use other substrates, derived from fatty acids, as fuel in their metabolic pathways. Unfortunately, this 'back-up' plan includes the release of ketoacids into the bloodstream, causing the pH of blood to drop, and diabetic ketoacidosis to develop. If not timely corrected by the injection of insulin, coma and death from acidosis will eventually occur.

The exact cause of T1D is still unknown. In most people with the disease, their bodies' own immune system mistakenly destroys the islets, as evidenced by the presence of autoantibodies directed against insulin, and islet-destructive T lymphocyte clones (reviewed in Ziegler et al. [1], and Rood et al. [2]). This is a process that originates through a combination of genetic factors - diabetes is

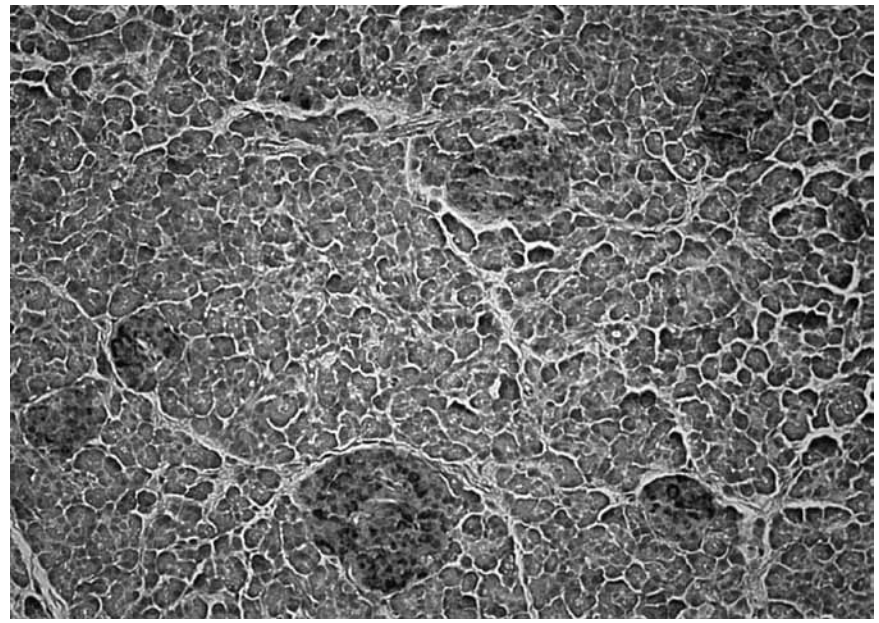

FIGURE 1: Light microscopy image (40x) of human pancreas showing islets, immunostained for insulin (red). (Color figure in appendix)

more prevalent in certain (Caucasian) families and is associated with certain human leukocyte antigen (HLA) subtypes (1, 2) - and environmental factors not yet fully understood. The resulting gradual loss of insulin-producing beta cells begins early in life and often leads to the diagnosis of T1D in childhood, hence the term 'juvenile-onset' diabetes.

Worldwide, there are many millions of diabetic patients. The incidence of T1D varies from 0.2-0.4% in unaffected families and can go up to 3%, 5%, and 8% if a child has an affected mother, father, or sibling, respectively (3-5). The number of young people developing T1D each year is currently estimated to be 17,100 in Europe and 14,700 in North America (6). These numbers accumulate to a prevalence of an estimated >2 million people with T1D in the US alone.

COMPLICATIONS OF THE DISEASE

Patients with the disease depend on subcutaneous injections of insulin for blood glucose control as a life-saving therapy to prevent diabetic ketoacidosis. The use of short-term and long-term acting insulin, or the use of an insulin pump for continuous release can nowadays almost mimic the natural release of insulin by the pancreas, as it would normally do in response to changes in blood glucose levels. However, the regulation of blood glucose values in diabetic

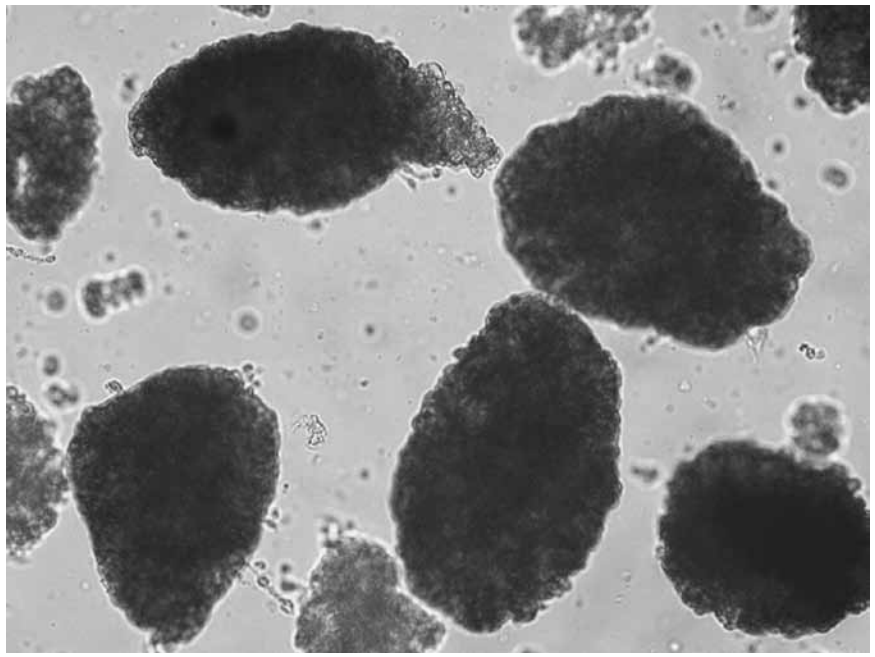

FIGURE 2: Human islets after isolation and purification, stained *in vitro* with dithizone (red). (Color figure in appendix)

patients cannot be perfect, and these slight metabolic imperfections can lead to devastating complications in the long-term. Because the disease may begin in childhood or adolescence, patients with T1D are at risk of developing complications after 40 or 50 years. These complications are mainly related to changes in large and small blood vessels that are sensitive to damage caused by elevated glucose levels. As a result, people with diabetes have an increased risk of dying (7), predominantly from heart attack or stroke (8). The damage to the small vessels found in the kidney, the retina, and around peripheral nerves eventually leads to kidney disease (diabetic nephropathy) (8-10), loss of vision (diabetic retinopathy) (9, 10), and loss of sensibility of hands and feet (diabetic neuropathy) (9), respectively. Unfortunately, attempts to prevent complications with intense insulin therapy programs increase the risk of severe hypoglycemia (11).

TRANSPLANTATION AS A CURE FOR DIABETES

The risk of complications of T1D has been a reason to desire beta cell replacement. The restoration of a patient's endogenous insulin production could fine-tune blood glucose control to that in a healthy individual, and would avoid the inconvenience of keeping strict schedules for the timing of meals, finger pricks to check glucose levels, and insulin injections. Several

investigations to achieve this are being undertaken, such as Tx of the pancreas or pancreatic islets, stem cell therapy (reviewed in Aguayo-Mazzucato and Bonner-Weir [12]) and abrogation of the autoimmune response that may possibly be followed by regeneration of beta cells (reviewed in Rood et al. [2]).

For restoration of beta cell function by Tx, two options exist - (i) whole pancreas Tx, or (ii) Tx of just the islets after a procedure to enzymatically isolate them from the exocrine tissue. With the Tx of the whole pancreas, 1-year success rates of greater than 80% have been achieved (reviewed in White [13]). After a successful pancreas transplant, glycemic control becomes comparable to that in healthy controls and some beneficial effects on the development of cardiovascular disease, retinopathy and neuropathy have been observed (13). Unfortunately, the potential beneficial effect on nephropathy has been counterbalanced by the need for immunosuppressive drugs that negatively affect kidney function (13). In addition, pancreas Tx is a major surgical operation, during which not only the blood vessels need to be connected to the patient's bloodstream, but also an outlet for the (redundant) exocrine digestive juices needs to be created. These exocrine secretions can be drained into either the intestine or the bladder. Leakage of this connection (anastomosis) can cause serious disease that sometimes requires re-operation. The risk of operative complications should be weighed heavily, since the Tx of the pancreas is not a directly life-saving procedure. Therefore, pancreas Tx is in most cases reserved for patients with end-stage diabetic nephropathy who also require a kidney transplant. They receive a pancreas at the time of kidney Tx.

The particular anatomy of the pancreas led to reasoning that only the 1-2% of tissue that produces insulin needs to be transplanted - islet Tx. Using collagenase to enzymatically digest the pancreas, islets can be isolated from the exocrine tissue. When a semi-automatic method for islet isolation was introduced in 1986 (14), the collection of human donor islets in consistent yields sufficient for clinical Tx became a reality (Figure 2). The resulting islet graft has a volume of less than 1mL. Theoretically, this graft can be injected as a cell transplant at one of many potential anatomic sites of the body, with avoidance of the risks of a major surgical operation and subsequent risks of leakage of an anastomosis. In current clinical practice, the liver is the site of choice for islet Tx. In normal non-diabetic conditions, insulin from the pancreas is secreted directly into the portal venous system and is metabolized largely in the liver (15). It was therefore reasoned that the liver might provide a physiologic environment for islets that would enable the effective use of the secreted insulin (16). Under local anesthesia after sedation, a catheter can be placed into the portal vein (the vein carrying blood from the intestines, pancreas and spleen to the liver) under ultrasound or fluoroscopic guidance (17, 18). The islets can then be infused through the catheter and are carried by the portal bloodstream into the liver. Although not entirely free from complications,

such as portal hypertension and bleeding, this minimally invasive procedure can be considered relatively safe and efficacious (18, 19).

THE CURRENT STATUS OF ISLET TRANSPLANTATION

The Tx of deceased human donor islets (allotransplantation [alloTx]) into the portal vein of diabetic patients has been followed by encouraging results (reviewed in Korsgren et al. [20], Merani et al. [21] and Hogan et al. [22]). A major breakthrough was forged in 2000 by the Tx of islets from more than one donor, and the introduction of an immunosuppressive protocol free of corticosteroids (23). Besides their immunosuppressive effect that prevents transplant rejection, corticosteroids are known to cause an increased need for insulin and therefore reduce the efficacy of the new islet graft.

However, a decade later some limitations of Tx into the portal vein have become obvious, and need to be considered in the further development of islet Tx as a treatment option for diabetes:

(i) Although considered a relatively safe procedure, the infusion of islets into the portal vein led to hemorrhage in 12% of transplant procedures and portal vein thrombosis in 4% (24).

(ii) An immediate loss of a large number of transplanted islets (estimated at 60-80%) through an inflammatory response known as the 'instant blood-mediated inflammatory reaction' (IBMIR) occurs immediately after infusion (20, 25). The loss of many islets through this inflammatory reaction is almost certainly a factor in the need for islets from more than one donor to achieve normal blood glucose values (normoglycemia) (23), and a major reason why it has proved difficult to consistently achieve normoglycemia in all transplanted patients (26).

(iii) Because the infused islets diffusely spread within the branches of the portal vein in the liver, biopsy of the site of the islets is not possible, and therefore the cause of loss of islet function, e.g., from IBMIR or acute rejection, cannot be fully assessed by histologic analysis.

(iv) The long-term results of the initial successful cases demonstrated that only 10% of patients were still insulin-free after 5 years (24). This means that in the majority of patients, there was a steady loss of normoglycemia, necessitating a return to insulin therapy. Because of this, the potential long-term beneficial effect on the complications of T1D has yet to be confirmed. Nevertheless, even a partially-functioning islet graft can greatly reduce the number of episodes of hypoglycemia (which is sometimes life-threatening) in patients who suffered from hypoglycemia unawareness before Tx (27, 28).

(v) The immunosuppressive drugs that a patient with an islet transplant needs to take to prevent rejection of the graft carry the risk of side-effects when used long-term. For example, calcineurin inhibitors, a powerful class of drugs that prevents rejection, are nephrotoxic, meaning that the kidney function, which is already compromised in diabetic patients because of diabetic nephropathy, may further decline after islet Tx and the administration of a calcineurin inhibitor (29). Therefore, immunosuppressive regimens that do not include these drugs still need to be developed for maintenance of renal function. Until then, islet Tx will be indicated only in patients who have disabling or life-threatening hypoglycemia unawareness or in patients who are already receiving immunosuppressive agents because of a previous kidney Tx (or who are about to receive a kidney graft).

(vi) The Tx of islets, especially when coming from multiple donors, puts the patient at risk of becoming allosensitized (detected as heightened panel reactive or anti-HLA antibodies) after the islet graft fails and immunosuppressive therapy is discontinued (30-32), although one study has suggested otherwise (33). This increases the risk of rejecting a subsequent islet or organ graft, if required.

(vii) The number of deceased human pancreas donors is insufficient to treat more than a small percentage of patients with T1D.

PIG ISLET XENOTRANSPLANTATION IN NON-HUMAN PRIMATES

Because of the shortage of human donor organs, alternative sources of cells and organs for Tx are being investigated. The Tx of pig islet into humans (xenotransplantation [xenoTx]) would offer the prospect of an unlimited number of islets, both for the initial transplant procedure, and, if necessary, for multiple subsequent transplant procedures. The methods for isolation of pig islets are well-established (34). It is also known that porcine insulin is able to regulate human blood glucose metabolism, as diabetic patients were treated with pig insulin for many years until human recombinant insulin was developed. Furthermore, although genetically more distant from humans than non-human primate (NHP) species, pigs as donors would be ethically more acceptable as a source of cells and organs for Tx than NHPs. Large-scale breeding of pigs (for our food-chain) is already established, and pigs grow to a size and weight comparable to a human adult within just a few months. Since the pig islets could be available at any time, the Tx could be electively scheduled after optimal preparation of the recipient patient. The current evidence is that pig islet Tx, even if associated with xenosensitization, would not lead to sensitization against alloantigens, and would therefore not compromise subsequent alloTx of a kidney (reviewed in Cooper et al. [35]).

The pig has the added advantage that it can be genetically-engineered to protect the source islets from insults such as IBMIR and immune-related responses. However, before applying pig-to-human islet xenoTx in patients with T1D, its safety and efficacy need to be scientifically proven, preferably in a NHP model. Encouraging results have been obtained in this model with the use of wild-type (WT, i.e., unmodified) pigs (36-39). Nevertheless, large numbers of pig islets need to be transplanted, likely because of the initial loss from IBMIR, and intensive immunosuppression is needed to prevent subsequent rejection. More and stronger immunosuppressive medications than needed to prevent allograft rejection have thus far been required to prevent rejection of islet xenoTx. These medications carry the risk of adverse effects that would severely limit the acceptance of islet xenoTx as a safe treatment for diabetes, and the need for safe yet efficacious immunosuppression is evident. In this respect, the potential use of WT pig islets seems limited. With genetic modification, human genes can be introduced into the pig islets that regulate and prevent IBMIR and islet rejection, thus reducing the need for intensive exogenous immunosuppressive therapy and its consequent side-effects.

While the studies resulting in this thesis were being undertaken, the criteria to be fulfilled from studies in NHPs before attempting a clinical trial of islet xenoTx were released by the International Xenotransplantation Association (40). In brief, normoglycemia in the absence of exogenous insulin injections is to be achieved for a period longer than 6 months in 5 of 8 NHP experiments, while using an immunosuppressive regimen that is safe, efficacious, and approved by regulatory authorities (40).

Aims

The research that is reported in this dissertation was aimed at improving the conditions for successful xenoTx of pig islets. The studies include those using an *in vitro* model and those carried out in preclinical large animal models, in order to advance this therapy toward a clinical option for the cure of T1D.

Aim 1 was to increase the understanding of the IBMIR, as this causes a major compromise to the efficacy of islet Tx into the portal vein. A review of the existing literature was undertaken (**Chapter 2**), and a simplified *in vitro* model of islet Tx was developed to investigate this immediate inflammatory response (**Chapter 3**). In this model, conditions of autotransplantation (autoTx), alloTx, and xenoTx were created and compared for induction of clotting, binding of antibodies, activation of complement, and damage to islet cells. Five different treatment strategies were applied to create insight into the mechanisms of IBMIR and to identify therapeutic options to minimize this event.

Aim 2 was to investigate the effect of the transgenic (Tg) expression of human CD46 (hCD46) on pig islets in a preclinical model of pig-to-diabetic NHP xenoTx (**Chapter 4**). hCD46 is a human complement-regulatory protein that is expressed on the cell membrane and prevents or reduces complement-mediated cell lysis (41). The hypothesis was that hCD46 reduces the islet loss from IBMIR and that the Tx of Tg islets results in long-term normoglycemia. The outcome of Tx with hCD46 Tg pig islets was compared with that of WT islets.

A number of the above-mentioned limitations of islet Tx are related to the site of implantation, which in current clinical practice is the portal vein; these include the risks of bleeding or thrombosis, the provocation of the IBMIR, and the impossibility of biopsying the graft to investigate the presence of rejection or the cause of graft failure. Since the islet graft is a cell transplant of only a small volume that does not require an anastomosis with the blood stream, it could theoretically be injected into many sites in the body. An alternative site for the liver may actually avoid the limitations of the portal vein.

Aim 3 was to investigate sites for islet Tx as an alternative for the portal vein. A review of the existing experience with different anatomical sites was conducted (**Chapter 5**). The gastric submucosal space (GSMS) was investigated as a novel

site for islet Tx in pigs (**Chapter 6**). The hypotheses were that (i) as the GSMS is accessible with the use of an endoscope through the patient's mouth and esophagus, this would allow injection of the islets as a minimally-invasive and quick procedure that would be of minimal discomfort to a diabetic patient, (ii) as the islets would not be injected directly into the blood stream, IBMIR should be avoided, and (iii) although the islets would not be in direct contact with blood, the GSMS provides a rich arterial supply of highly-oxygenated blood to enable islet survival.

The experiments in ***Aim 2*** were performed with reduced immunosuppressive therapy in comparison with previous studies (36, 38), while the (chronic) use of corticosteroids or calcineurin inhibitors was avoided. However, a monoclonal antibody against CD154 (anti-CD154 mAb) for costimulatory blockade was used. This anti-CD154 mAb possesses great potential to inhibit the immune response against xenoantigens (42), but, in view of their potential thrombogenic effects (43-46), they are unlikely to be approved for clinical use. The need for safe immunosuppression is obvious. At the University of Pittsburgh Medical Center (47, 48) and other centers (49, 50), excellent results have been obtained with the use of alemtuzumab (anti-CD52 mAb, Campath-1H) for induction therapy in clinical alloTx, including pancreas Tx (51, 52) and islet Tx (53, 54). Alemtuzumab may allow for a low level of maintenance immunosuppression (48). With a strong induction agent, such as alemtuzumab, control of the T lymphocyte response with a weaker, but clinically-acceptable, agent for costimulation blockade (such as CTLA4-Ig) can be hypothesized. However, in many NHP species CD52 is expressed on lymphocytes as well as on red blood cells (RBC) (55), leading to life-threatening hemolysis when this agent was used in these animals (Prof. Gerard Wagemaker, Erasmus University Rotterdam, personal communication).

Aim 4 was to identify NHPs in which CD52 is not expressed on RBC, to perform a dose-efficacy study with alemtuzumab, and to investigate its effects on the NHP immune system (**Chapter 7**), with a perspective of a future alemtuzumab-based immunosuppressive regimen that prevents xenorejection in NHPs, and in addition would be safe for patients.

In the near future, islet xenoTx may become the first clinical application of xenoTx. Consideration is also being given to how other potential applications of xenoTx can be justified. For example, patients severely ill from fulminant liver failure may benefit from a pig liver graft as 'a bridge' until a human donor liver becomes available (56, 57). The immature immune system of infants possibly provides an opportunity for Tx with less susceptibility to rejection, or even the development of tolerance (58). In pediatric heart transplant programs, heart Tx across the ABO-blood group barrier is possible (59), while in adults this procedure has to be undertaken more cautiously. The development of antibodies against blood groups other than one's own (e.g., a person with

blood group A will develop natural antibodies against blood group B) does not occur until after the first few months of life, providing a 'window of opportunity' for an ABO-incompatible organ transplant during this period of time without the risk of hyperacute rejection (59). Moreover, organ Tx during this period can result in the development of B cell tolerance to the incompatible blood group antigen(s), which means that antibodies against the donor blood group never develop. The development of natural antibodies against carbohydrate structures on pig cells that resemble human blood group antigens follows a similar pattern (60). However, less is known about the development of the immature cellular immune system in infants (particularly relating to T lymphocytes) and its relation to organ Tx.

Aim 5, therefore, was to investigate the natural development of T lymphocyte subsets in healthy infant baboons (**Chapter 8**). The neonatal and infant immune system of laboratory mice has been thoroughly studied (reviewed in [61]). About the immune system early in the life of humans and NHPs, less is known (62-65). T lymphocyte immune responses in healthy infant baboons were compared with those in infant baboons exposed to an allograft or pig xenograft in the presence or absence of immunosuppressive therapy (**Chapter 8**). These studies were aimed at determining whether a 'cellular window of opportunity' for tolerance induction after infant (xeno)Tx is likely to exist.

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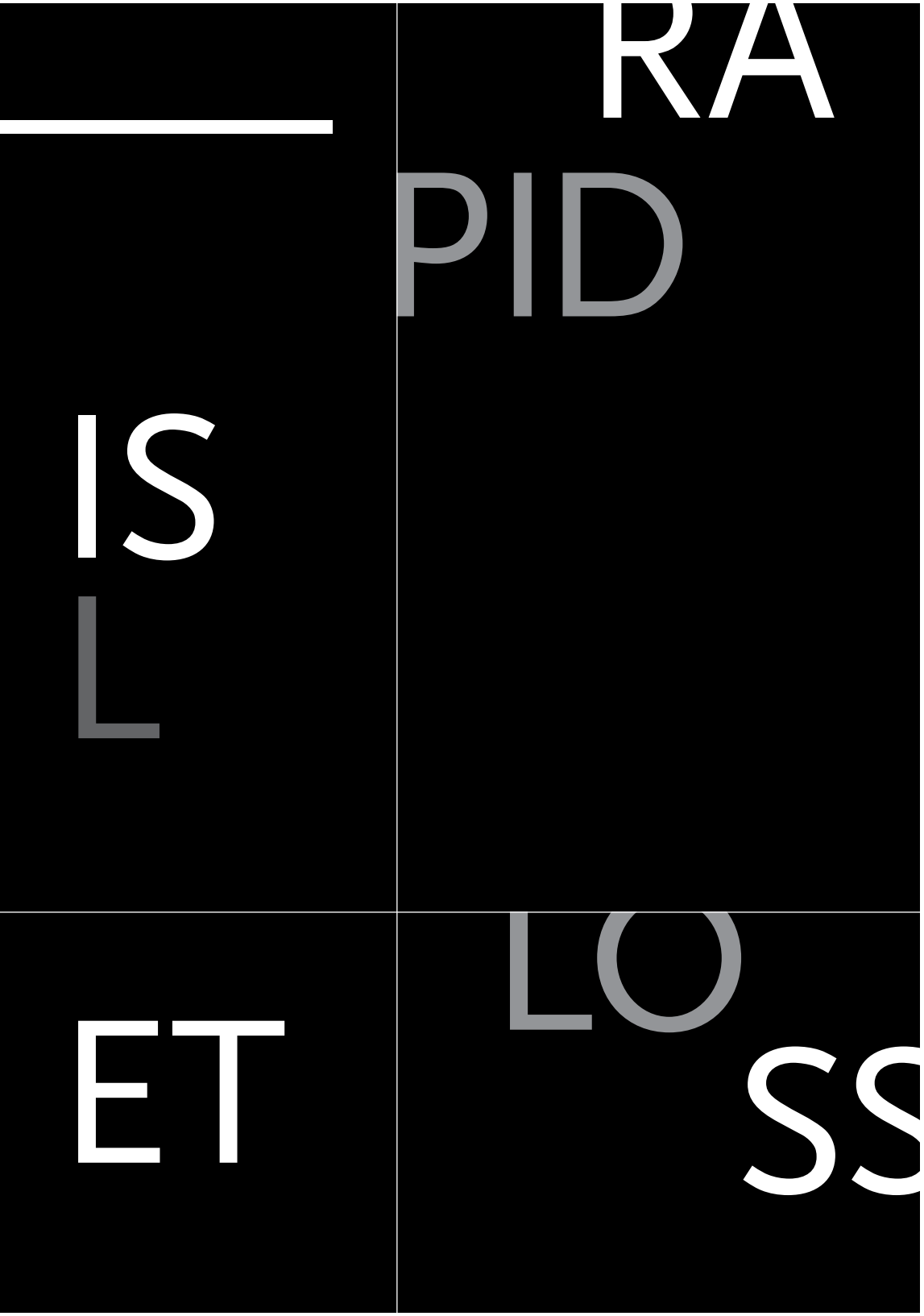

CHAPTER 2

Rapid loss of intraportally transplanted islets: an overview of pathophysiology and preventive strategies

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ABSTRACT

Islets isolated from multiple pancreas donors are often necessary to achieve normoglycemia in patients with T1D treated by islet alloTx. This increases the burden on the limited pool of donor organs. After infusion into the portal vein, a substantial percentage of islets are lost in the immediate post-transplant period through an inflammatory response termed the 'instant blood-mediated inflammatory reaction' (IBMIR). IBMIR is equally, if not more of a problem after islet xenoTx, e.g., using pig islets in NHPs. Coagulation, platelet aggregation, complement activation, and neutrophil and monocyte infiltration play roles in this reaction. IBMIR is potentially triggered by islet surface molecules, such as tissue factor (TF) and collagen residues that are normally not in direct contact with the blood. Also, stress during the islet isolation process results in the expression and production of several inflammatory mediators by the islets themselves. The potential mechanisms involved in this rapid graft loss and treatment options to reduce this loss are reviewed. Preventive strategies for IBMIR can include systemic treatment of the recipient, pre-conditioning of the isolated islets, or, in the case of xenoTx, genetic modification of the organ-source pig. Pre-conditioning of islets in culture by exposure to anti-inflammatory agents or by genetic modification harbors fewer risks of systemic complications in the recipient. The future of clinical islet Tx will, at least in part, depend on the success of efforts made to reduce rapid graft loss, and thus allow islet Tx to become a more efficient therapy by the use of single donors.

Introduction

The Edmonton protocol for clinical islet Tx can successfully restore endogenous insulin production and glycemic stability in patients with T1D. However, insulin independence is usually not sustained despite islet infusions from two or more donors (1). The current supply of islets from deceased human donors will almost certainly never be able to meet the demand. XenoTx, using the pig as the source of islets, could potentially resolve this problem.

One of the problems limiting the success of intraportal islet Tx is early and rapid loss of a significant portion of the graft. Its occurrence suggests a role for non-immunological as well as innate immunological factors (2). Coagulation, platelet aggregation, complement activation, and neutrophilic granulocyte and monocyte infiltration have been reported to be involved in a reaction termed the IBMIR (2). This results in what has previously been described as 'early graft loss' or 'primary non-function'. In clinical islet alloTx, it is estimated that up to 70% of the transplanted mass is lost in the early post-Tx phase (3). IBMIR is equally, if not more of a problem after porcine islet Tx, in NHP (4). Indeed, it may be the single most important barrier to successful islet Tx today.

Rapid islet loss can be associated with a peak in serum C-peptide levels, indicating islet disruption, which has been observed in clinical alloTx (5) as well as in our own pig-to-NHP preclinical model (Figure 1). It has to be overcome if adequate and stable graft function is to be achieved. In this review, the potential mechanisms leading to destruction of the islet beta cells will be discussed, and strategies to overcome this loss will be considered. Preventive approaches for IBMIR can be aimed at (i) systemic treatment of the recipient, (ii) pre-conditioning of the islets *in vitro*, including genetic alteration, or (iii) in the case of xenoTx, genetic modification of the organ-source pig. This latter approach could provide an inexhaustible source of islets. Indeed, the ability to modify the pig to provide protection of its islets from IBMIR might eventually prove pig islets superior in this respect to human donor islets. Concepts derived from NHP alloTx and xenoTx studies that may possibly indicate future approaches to the control of IBMIR are included in this overview.

Pathophysiology of Early Islet Graft Loss

COAGULATION AND PLATELET ACTIVATION

Almost a decade ago, a major incompatibility between islets and whole blood was demonstrated for the first time (6). In an *in vitro* system, using tubing loops as a model for a blood vessel, islets became trapped in clots, indicating a dominant role for the coagulation system and platelets (6). This was confirmed *in vivo* when thrombosis and necrotic islets were seen in liver biopsies 2 days after pig islets had been transplanted into the portal vein of a NHP (7). When serum alone or serum with neutrophils, rather than whole blood, were used in this looping assay, full-blown IBMIR was not seen (8), suggesting that whole blood, including its coagulation factors, is a pre-requisite, making IBMIR inherent to Tx into the portal vein.

Coagulation can be activated through the intrinsic or extrinsic activation pathway (Figure 2). In intraportal islet Tx, it is plausible that both pathways become activated. The intrinsic pathway is activated by collagen and other negatively charged molecules on the islet surface that normally are not in contact with blood (2). The extrinsic pathway is activated by TF. Isolated islets express TF on alpha and beta cells and secrete TF together with insulin and glucagon (5). TF has also been detected on the ductal structures that contaminate islet preparations (9). The clinical outcome of islet Tx is directly related to the extent of TF expression (10). It is evident that anticoagulation has to be one of the cornerstones of the therapeutic approach to prevent or reduce IBMIR.

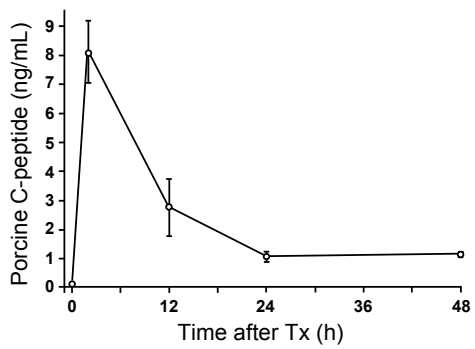

FIGURE 1: Porcine C-peptide levels in recipient serum in the early phase after intraportal islet xenoTx in cynomolgus monkeys. Blood samples for porcine C-peptide assay were taken at 2, 12, 24, and 48 h after Tx (n = 3). In two monkeys, an additional sample was taken at 60 or 90 min after Tx, showing C-peptide levels of 12 and 10 ng/ml, respectively. These observations, combined with severe hypoglycemia requiring intravenous dextrose infusion, represent quick and massive islet disruption after Tx.

After pig-to-NHP islet Tx, the extent to which coagulation is activated can be even greater than after human islet alloTx, as several molecular incompatibilities between the primate and pig (anti)coagulation systems exist (11). In particular, TF pathway inhibitor and thrombomodulin expressed on porcine cell membranes fail to regulate primate coagulation mechanisms; as a consequence, a failure of anticoagulation occurs (12, 13).

Several pathways lead to platelet activation. If collagen residues on the isolated islets are exposed to blood, they can bind soluble von Willebrand factor (vWf). Platelets possess intra-membrane glycoproteins that serve as receptors either for collagen directly (GpIIb/IIIa, GpIV, and GpVI) or for collagens covered by vWf (GpIb, GpIIb/IIIa), and that are integral to their activation. A second mechanism of platelet activation is through coagulation, with thrombin (via binding to GpIb) as the most important stimulator (14). In *in vitro* studies, platelets were consumed after coagulation had been activated (15). After islet Tx, coagulation-induced platelet activation would appear to play an important role in IBMIR (15). Furthermore, activated platelets induce more coagulation, and so the cycle is continued (11).

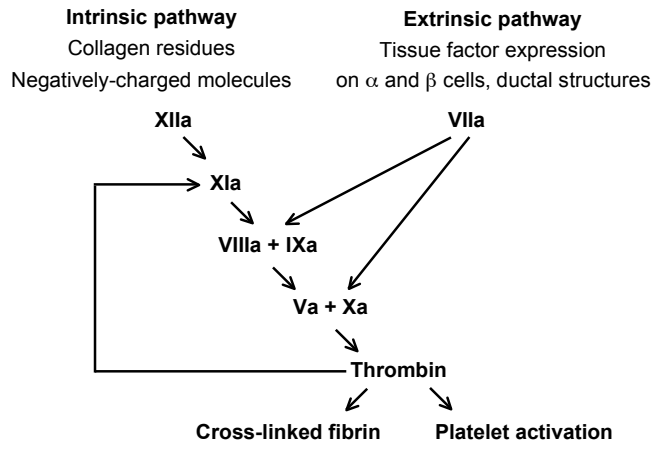

FIGURE 2: Schematic representation of the coagulation pathways. For simplicity, only the activated clotting factors are shown, indicated by 'a'. Briefly, in IBMIR, coagulation is initiated when islet-expressed tissue factor (TF) is exposed to the blood. TF then complexes with VIIa and enhances its activity. This sequence of coagulation activation is known as the extrinsic pathway. This complex of VIIa/TF activates factors IXa and Xa, which mediate the conversion of prothrombin into the active thrombin. Nevertheless, the small quantity of thrombin formed is sufficient to activate XIa, which reinforces thrombin generation by activating the intrinsic pathway. Furthermore, the intrinsic pathway can be activated by collagen residues or other negatively charged molecules on the islet surface. Thrombin, a potent platelet activator, cleaves fibrinogen into fibrin monomers, and activates the coagulation factor that cross-links fibrin monomers into an insoluble thrombus (XIIIa, not shown).

As platelet activation can be thrombin-independent, the addition of platelet inhibitors to the available anticoagulants may prove beneficial. However, therapy must be cautious to prevent uncontrolled bleeding, which is a known complication of intraportal islet infusion (16). However, some platelet activation around the islets may have a beneficial effect on engraftment and revascularization through the production of several growth factors that stimulate angioneogenesis, such as vascular endothelial growth factor (17).

PREFORMED CIRCULATING ANTIBODIES AND COMPLEMENT ACTIVATION

Complement can be activated through the classical pathway by antibodies binding to their targets and resulting in cytotoxicity (Figure 3). However, in initial *in vitro* studies using intact human or pig islets, no antibody deposition could be detected on their surface after the contact with whole human blood (6, 18). It was therefore concluded that hyperacute rejection, in which preformed antibodies bind to the graft and activate complement, resulting in cell lysis, is not a contributory mechanism in IBMIR. The massive complement activation that was observed to take place in IBMIR occurs possibly through the alternative pathway (6, 18, 19). The mechanisms through which isolated islets activate the alternative pathway, and the involvement of the lectin pathway, have not yet been elucidated.

In contrast to these initial reports, recent results indicate that the outcome of clinical islet alloTx is inferior when preformed antibodies are present, e.g., when there are high antibody titers against foreign HLA (20). In addition, autoantibodies in patients with T1D have been demonstrated to bind allo-islet beta cells and fix human complement (21). Patients with high pre-transplant autoantibody levels did not achieve normoglycemia after islet alloTx (1, 22). Therefore, a role for circulating antibodies in IBMIR cannot be excluded. Islet donor and recipient are always matched for ABO blood group, but HLA matching is difficult, particularly if multiple donors are required. Clinical islet alloTx has proven to be most successful when a patient with a negative serum cross-match against the donor, or only a low level of panel-reactive antibodies against donor HLA types, undergoes Tx (23–25). These observations may justify a new evaluation of the role of preformed antibodies in IBMIR. Absence of such antibodies might allow for a smaller initial loss of islets in the first hours after portal infusion, and may be a factor in the achievement of normoglycemia after the infusion of islets from a single donor (23).

In organ xenoTx, the antibodies mainly responsible for hyperacute rejection are directed to the galactoseα1,3galactose (Gal) epitope on pig endothelium (26). Adult pig islets hardly express Gal on their surface (4,27), and therefore anti-Gal antibody binding is minimal or absent (28). The introduction of pigs that are homozygous for α1,3-galactosyltransferase gene-knockout (GT-KO) might

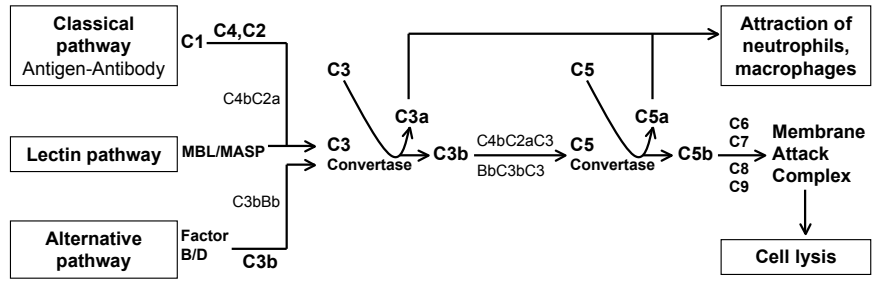

FIGURE 3: Schematic representation of the complement cascades. MBL = Mannose binding lectin; MASP = MBL-associated serine protease.

therefore not be of great benefit for graft survival of intraportally transplanted adult pig islets (29), although Gal is abundantly expressed on WT exocrine cells that typically contaminate the islet graft preparation (28). An antibody-mediated inflammatory response against Gal on the contaminating exocrine tissues may be detrimental to the islets as well (28). In contrast to adult islets, fetal or neonatal pig islets express Gal, and therefore the Tx of islet-like cell clusters from GT-KO piglets will almost certainly be advantageous.

The influence of antibodies against epitopes other than Gal is not clear. Human and NHP sera can contain other preformed antibodies that may account for significant binding to pig islets (19). Major targets for human antibodies include sialic acids, in particular *N*-glycolylneuramic acid (19), which, however, is unlikely to play a role in pig-to-NHP islet Tx, as both pigs and NHPs express this oligosaccharide. The contribution of these and other anti-nonGal antibodies to IBMIR is largely unknown and requires investigation.

INFILTRATION OF INFLAMMATORY CELLS: NEUTROPHILIC GRANULOCYTES AND MACROPHAGES

Within 1h, platelet consumption, coagulation, and complement activation are followed by infiltration of the islets by $CD11b^+$ neutrophilic granulocytes and macrophages (30). B or T lymphocytes are not seen at this stage (30). There are plausible explanations of why this infiltration is part of IBMIR as multiple interactions between coagulation, complement, and inflammatory cells are known to occur. For example, activated platelets upregulate their surface expression of P-selectin, which is their primary receptor for neutrophil and monocyte interactions (31). Thrombin stimulates protease-activated receptors that are present on granulocytes and monocytes, and their signaling pathways lead to enhanced cytokine production (32). TF, fibrin, and fibrinogen may also have a direct activating effect on macrophages (33). The effects of coagulation on inflammation after Tx have recently been reviewed (34). In addition, activated

portal endothelial cells produce platelet-activating factor, which is a potent neutrophil chemoattractant (35). Furthermore, the complement activation products, soluble C3a and C5a, serve as highly potent chemoattractants for neutrophils and macrophages, thereby catalyzing the inflammatory reaction (6). In an *in vitro* model, anticoagulation prevented cellular infiltration of islets to a great extent, and provided confirmation of these interactions (15, 36).

Evidence is accumulating that isolated islets are in a proinflammatory state after they have been subjected to the isolation procedure, in which they are exposed to mechanical and oxidative stress. They express a great variety of inflammatory mediators, including monocyte chemoattractant protein-1 (MCP-1), IL-1β, TNF-α, IFN-γ, IL-6, and IL-8 (37–41). The extent of production of MCP-1 correlates with clinical graft survival (41). Islet-expressed cytokines and chemokines are potent chemoattractants of inflammatory cells (42). In rodent experiments, intra-islet levels of IL-1, TNF-α, and MCP-1 were further upregulated after Tx (43), and played a role in the high rate of early graft failure (44). Inflammatory cytokines induce beta cell damage through activation of several intracellular stress-signaling pathways. Recently, the importance of the p38 pathway and the pathway mediated by c-Jun NH2-terminal kinase after intraportal islet Tx was demonstrated (40, 45).

Infiltrating cells are directly cytotoxic to islet cells. Degranulation of neutrophils can lead to enzymatic digestion of target cells, and both neutrophils and macrophages are major phagocytes. Secondly, inflammatory cytokines lead to apoptosis of islet cells, mainly by TNF-α signaling pathways (46) and upregulation of Fas expression (47). These pathways induce apoptosis through the activation of nuclear factor-κB-regulated apoptotic genes and activation of caspases (47,48). Apoptosis may account for a substantial part of the early graft loss in islet Tx (49). A third effect of neutrophil and macrophage involvement in IBMIR is their role of antigen-presentation to T cells. T cells are known effector cells of cellular rejection of islets after the initial inflammatory stage (50). Therefore, IBMIR is likely to induce or increase a subsequent adaptive immune response.

Strategies to Prevent Early Graft Loss

If early graft loss after intraportal islet Tx is to be reduced, interventions can be directed against the various components of IBMIR or, ideally, against all components by a single agent, if such an agent exists. The interventional strategies that have been investigated are based on (i) systemic treatment of the recipient, (ii) pre-treatment of the isolated islets before Tx, or (iii) genetic alteration of the islets, either *in vitro* or through genetic modification of the source pigs. The first option harbors the risk of systemic complications, especially that of bleeding when anticoagulation or anti-platelet drugs are involved (16). In recent years, therefore, to achieve a local effect at the optimum site, many efforts have been made to pre-treat or genetically modify the islets before Tx. These various strategies were aimed mainly at reducing coagulation, complement activation and inflammation. Although Tx of islets under the kidney capsule is not associated with IBMIR, this model has been used to provide evidence that some anti-inflammatory agents can reduce the inflammatory response to islet Tx. Tables 1-3 summarize the results of the published studies. Most of the strategies that have been employed are also briefly discussed below.

TREATMENT OF THE ISLET TRANSPLANT RECIPIENT

In current practice, islets are transplanted in heparinized medium to prevent coagulation (Table 1). In *in vitro* studies, the substitution of heparin by low molecular weight dextran sulfate (LMW-DS) blocked IBMIR to a greater extent (36,51). In *in vivo* rodent studies, LMW-DS treatment of the recipient significantly prolonged the survival of intraportally transplanted islets (51), an effect possibly related to the additional complement-inhibitory property of LMW-DS. However, large animal studies have not yet fully confirmed these observations. In our pig-to-NHP islet Tx experiments, LMW-DS treatment of monkeys only had a minimal effect on complement activity, but reduced IBMIR to a limited extent, likely through its anticoagulation properties (4).

Another anticoagulant, Nacystelyn, a derivative of *N*-acetylcysteine, has recently been shown to prevent islet-induced coagulation *in vitro* (52). In addition, it has anti-inflammatory and anti-oxidant properties that may be valuable in inhibiting IBMIR (52).

Table 1 Treatment options for the islet transplant recipient.

Agent	Mechanism	Experimental Experience (reference)	Clinical Experience / Applicability
Heparin	Anticoagulation	-	In current practice, islets are infused in heparinized solution
Low molecular weight dextran sulfate	Anticoagulation Complement inhibition	*In vitro:* Prevention of all aspects of IBMIR in tubing loops model (36, 51) *In vivo:* Adequate anticoagulation but limited complement inhibition in monkeys (4, 36). Prolonged survival of pig islets transplanted IP in diabetic mice (51). Reduction of IBMIR in pig-to-NHP islet Tx (4)	Clinical trials in HIV patients in which the antiviral effect of dextran sulfate is tested (71).
Nacystelyn (*N*-acetylcysteine derivative)	Anticoagulation Anti-inflammatory & anti-oxidant effects	*In vitro:* Prevention of islet-induced coagulation and platelet comsumption in tubing loops model (52)	*N*-acetylcysteine was tested in clinical trials in liver Tx to prevent ischemia-reperfusion injury (72, 73)
Melagatran	Anticoagulation via thrombin inhibition	*In vitro:* Anticoagulation via tubing (15) loops model	Clinical trials for anticoagulation in deep venous thrombosis and atrial fibrillation (74)
Anti-TNF-α monoclonal antibody (Infliximab)	Binding to TNF-α prevents stimulation of its receptor	-	No clinical difference in a randomized clinical trial in islet Tx (53)
15-Deoxyspergualin	Blockade of PIC production via NF-κB inhibition	*In vivo:* Long-term normoglycemia after monkey islet alloTx (54)	Clinical trials in combined kidney/islet Tx (75)
Activated Protein C	Anticoagulation Fribrinolysis NF-κB inhibition	*In vivo:* Reduction of coagulation, PIC release, cell infiltration, and insulin dumping in syngeneic mice after IP islet Tx (31)	Clinical trials for treatment of patients with severe sepsis (76)
Statins	Inhibition of thrombin formation Immunomodulatory & anti-inflammatory effects	*In vivo:* Dogs transplanted with a marginal autologous islet mass achieved and maintained normoglycemia (55)	Clinical trials in heart and kidney Tx (77, 78)

PIC = Pro-inflammatory cytokines; IP = Intraportal(ly)

Treatment of the recipient with either an anti-TNF-α mAb (infliximab) or 15-deoxyspergualin has been demonstrated to reduce the inflammatory response (53,54). 15-Deoxyspergualin treatment of recipient monkeys led to normoglycemia for >1 year following alloTx (54), though infliximab did not improve the outcome of clinical islet alloTx (53).

Statins are of current interest in Tx because of their anticoagulant and anti-inflammatory properties, combined with a low risk of side effects. Besides their administration to the recipient, exposure of the isolated islets to statins in culture or, in xenoTx, treatment of the donor pig, may result in improved islet graft survival (55–57).

PRE-TREATMENT OF THE ISOLATED ISLETS PRIOR TO TRANSPLANTATION

Pre-treatment of isolated islets has mainly been aimed at reducing their pro-coagulant activity, e.g., by downregulation of TF expression with nicotinamide (58), or at reducing the detrimental effects of inflammation (Table 2). A natural inflammatory regulator is α1-antitrypsin, a major protease inhibitor that inhibits the enzyme activity of neutrophil elastase and thrombin. *In vitro*, in the presence of α1-antitrypsin, mouse islets were protected against the effect of the cytokines, IL-1β and IFN-γ, by means of greater viability, a 40% reduction in nitric oxide production, and greatly diminished TNF-α production (59). Moreover, administration of human α1-antitrypsin to recipient mice doubled the period of islet survival, and would be a safe approach in a clinical setting (59).

Anti-oxidant supplementation to the islet culture medium for scavenging of oxygen radicals helped human islets to reduce their inflammatory state, seen by reduction of cytokine and MCP-1 expression (37, 60). When transplanted into mice, a reduced mass of anti-oxidant-treated islets were able to restore normoglycemia, compared with untreated islets (61).

Heme oxygenase-1 is the rate-limiting enzyme in the degradation of heme into biliverdin, carbon monoxide, and free iron. All these degradation products have been shown to have cytoprotective and anti-inflammatory effects (62, 63). These beneficial effects can be obtained by treating islets with protoporphyrins or carbon monoxide, which has been reported to lead to allograft survival for >100 days when islets were transplanted under the kidney capsule of mice (46, 63). Heme oxygenase-1 induction reduced cytokine production and macrophage infiltration in the peri-Tx period (62).

For many years, it has been a desire and a challenge to 'hide' islets from the immune response by encapsulation. An advanced method for islet surface engineering has recently been developed, namely by covering the islets with polyethylene glycol (64). The capsule around the islets is ultra-thin, and allows them to be transplanted intraportally (65). It is tempting to assume that this might also have a protective effect against IBMIR, e.g., by blocking coagulation around the islets. Indeed, polyethylene glycol-modified porcine islets were resistant to human serum cytotoxicity, and were better able to restore and maintain normoglycemia when transplanted intraportally into diabetic mice, compared with controls (64). However, it is unclear to what extent polyethylene glycol can protect islets against inflammatory molecules such as cytokines and oxygen radicals.

GENETIC MODIFICATION OF ISLETS

Most *in vitro* genetic manipulations of human islets were aimed at the induction of genes with an anti-apoptotic effect (Table 3). The overexpression of X-linked inhibitor of apoptosis protein is the most promising to date, possibly because expression of this protein blocks the effector phase of apoptosis after multiple apoptosis-triggering pathways have converged (66). It reduced the human islet mass that was needed to treat diabetes successfully in mice by 40% (66).

In vitro anti-oxidant treatment of isolated islets allowed them to recover from stress and inflammation induced during the isolation procedure (37, 60). Scavenging of oxygen radicals at the time of their production during the isolation process might be more effective, and therefore the overexpression of anti-oxidant genes in islets has been investigated (67, 68). A reduced marginal

Table 2 Options for pre-treatment of the islets prior to transplantation.

Agent	Mechanism	Experimental Experience (reference) *In Vitro*	*In Vivo*
α1-Antitrypsin	Complement inhibition Blockade of PIC production	Resistance against PIC in culture (59) Reduction of islets' inflammatory state (59)	Long-term normoglycemia and less cell infiltration observed in diabetic mice transplanted with allo-islets under the kidney capsule (59)
Nicotinamide	Downregulation of TF expression on isolated islets	Reduction of activation of coagulation in tubing loops model (58)	-
Anti-oxidants	Scavenging of reactive oxygen species produced during isolation stress	Dose-dependent reduction of MCP-1 and PIC release (37, 60)	Diabetic mice transplanted with a marginal human islet mass under the kidney capsule achieved normoglycemia (61)
Statins	Protection against ischemia-reperfusion injury Inhibition of pro-apoptotic pathways	Dose-dependent increase in islet survival and viability in culture (56)	Diabetic mice transplanted IP with a marginal human islet mass achieved and maintained normoglycemia (56)
Heme oxygenase-1 induction	Anti-inflammatory effects via p38 MAPK-dependent pathway	Resistance against PIC in culture (46)	Less PIC production and monocyte infiltration in mice allo-islets transplanted under the kidney capsule (62)
Lisofylline	Anti-inflammatory effects by reduction of PIC production	Resistance against PIC in culture (79) Improvement of islet metabolism (79)	Reduction of the human islet mass needed to restore normoglycemia in diabetic mice (79)
Active vitamin D $1\alpha,25(OH)_2D_3$	Anti-inflammatory effects by induction of protective gene expression	Resistance against PIC and oxidative stress in culture (80)	-
Polyethylene glycol (PEG) surface engineering	Covering of islets to prevent direct exposure to blood	Resistance of porcine islets against human serum cytotoxicity (64)	Diabetic mice transplanted IP with a pig islet mass achieved normoglycemia (64)

PIC = Pro-inflammatory cytokines; IP = Intraportal(ly); MAPK = mitogen-activated protein kinase

Table 3 Potential genetic modifications of isolated human islets or of donor mice and pigs.

Genetic Modification	Mechanism	Experimental Experience (reference) *In Vitro*	*In Vivo*
Overexpression of anti-apoptotic genes	Adenoviral or protein-mediated overexpression of 'protective' genes, A20, Bcl-2, Bcl-xl, XIAP	Resistance against PIC and hypoxia-induced apoptosis in culture (66, 81-83)	Inconsistent results in models of human or mice islets transplanted under the kidney capsule of diabetic mice. Best results with XIAP (66)
Anti-oxidant transgenic mice	Transgenic overexpression of superoxide dismutase or glutathione peroxidase	Maintained islet viability after oxidative stress in culture (67)	Diabetic mice transplanted with a marginal islet mass under the kidney capsule achieved normoglycemia (67)
Anti-oxidant overexpression in human islets	Adenoviral-mediated overexpression of superoxide dismutase	Increased resistance against nitric oxide (68)	-
hCD39 transgenic mice	Transgenic overexpression of a thromboregulatory molecule	Reduction of coagulation after exposure to whole human blood (69)	-
hDAF / hCD59 overexpression on pig islets	Adenoviral-mediated or transgenic introduction of complement-regulatory proteins	Resistance against human serum cytotoxicity (70)	-

PIC = Pro-inflammatory cytokines; h = human; XIAP = X-linked inhibitor of apoptosis protein

islet mass from Tg mice expressing superoxide dismutases or glutathione peroxidase resulted in normoglycemia when transplanted under the kidney capsule of diabetic mice (67). Human islets transfected with anti-oxidant genes proved increasingly resistant to nitric oxide-induced injury *in vitro* (68).

If pigs are to serve as islet donors for patients, genetic modification of the pig would appear to be essential to overcome incompatibilities between pig and human coagulation and complement systems. To reduce the detrimental effects of IBMIR, it is essential that each genetic modification be expressed in the cells of the isolated islets. Islets from mice and pigs Tg for coagulation and complement-regulatory proteins have been shown to be less susceptible to the effects of coagulation and cytotoxicity *in vitro* (69, 70). Moreover, additional genetic alterations may help blocking several pathways involved in the early destruction of islets. For example, pigs Tg for human TF-pathway inhibitor may offer the islets protection from TF-induced coagulation, and pigs Tg for one or more anti-oxidant or anti-inflammatory genes may offer protection against rapid loss.

Conclusions

The initial loss of a large number of islets after Tx into the portal vein is caused by an inflammatory reaction composed of activation of coagulation and complement, platelet adhesion, and involvement of neutrophils and macrophages. Prevention or minimization of this reaction would seem to be essential, and could result in successful clinical Tx with a lower islet mass, thereby contributing to better utilization of the available human donor pancreases. Even in xenoTx, where the number of islets is limitless, IBMIR must be minimized if islet Tx is to become a practical therapeutic option for T1D.

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ISLETS BLOOD

CHAPTER 3

Early islet damage after direct exposure of pig islets to blood – has humoral immunity been underestimated?

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ABSTRACT

Currently, islet Tx as a cell therapeutic option for T1D occurs via islet injection into the portal vein. Direct contact between islets and blood is a pathophysiological 'provocation' that results in the 'instant blood-mediated inflammatory reaction' (IBMIR), and is associated with early islet loss. However, the nature of the various insults on the islets in the blood stream remains mostly unknown. To gain insight into the mechanisms, we utilized a simplified *in vitro* model in which islets were exposed to blood in different clinically relevant, but increasingly challenging, autologous, allogeneic and xenogeneic combinations. Irrespective of the blood type and species compatibility, islets triggered blood clotting. Islet damage was worse as islet and blood compatibility diminished, with substantial islet injury after exposure of porcine islets to human blood. Islet damage involved membrane leakage, antibody deposition, complement activation, positive staining for the membrane-attack complex and mitochondrial dysfunction. Islet damage occurred even after exposure to plasma only, and specific complement inactivation and neutralization of IgM substantially prevented islet damage, indicating the importance of humoral immunity. Efficacious measures are needed to reduce this injury, especially in view of a potential clinical use of porcine islets to treat diabetes.

Introduction

Islet Tx can successfully restore glycemic stability in patients with T1D (1). The fate of isolated islets infused into the portal vein is, however, determined by a number of damaging events, occurring as early as during islet injection. Together, these early factors are estimated to cause a loss of 70% of the transplanted islet mass (2). As a consequence, islets obtained from multiple organ donors become necessary in the majority of recipients to reach a sufficiently functional islet mass. Roles for cold centrifugation in the purification process (3), low oxygen tension of the portal venous blood (4), an active innate immune system including Kupffer cells (5) and the activation of the IBMIR (6) have been postulated. Nonetheless, the extent of the islet damage and the mechanisms involved early after islet injection in the blood stream, are not yet clearly understood.

The availability of human organ donors does not meet the increasing demand for human organs to cure severely invalidating and life-threatening diseases and drastically limits the development of programs of islet Tx. To this aim, the employment of porcine islets for clinical use is currently under investigation, and may become a therapeutic option in the near future (7). A better understanding of the events that occur when islets are in contact with blood, particularly in view of the possible use of xenogeneic islets, is therefore urgently needed.

Experimental *in vivo* studies have shown that an intensive inflammatory reaction occurs when islets are exposed to whole blood. The resulting islet cell damage is reflected by an early non-physiological peak in circulating C-peptide levels, which we and others have observed (8, 9). Some insights into the mechanisms that initiate IBMIR and early islet loss have been provided, such as the involvement of the coagulation and the complement systems (6). Multiple treatment options to circumvent these problems have been proposed and investigated (10-12), but promising *in vitro* results have thus far not been converted into successful prevention of early graft loss *in vivo*.

The aim of our study was to set up a simplified *in vitro* test to investigate the events that occur following contact of islets with whole blood or plasma, in order to identify approaches that minimize early islet loss *in vivo*.

Pig or human islets were selectively exposed to autologous, allogeneic or xenogeneic blood. We found that islets triggered blood clotting regardless of the combinations, whereas islet damage was greater in xenogeneic combinations than in autologous and allogeneic settings. Prevention of blood clotting (by low molecular weight dextran sulfate, LMW-DS) and targeting TF by nacystelyn (NAC), were not sufficient to prevent islet loss, whereas a specific complement inhibitor (compstatin) and anti-IgM antibodies efficiently reduced islet damage. These new insights may warrant more efficient protection of pancreatic islets in the peri-Tx phase.

Materials & Methods

SOURCES OF HUMAN AND PORCINE ISLETS

Human deceased donor pancreata (n=11) were obtained from the Center for Organ Recovery and Education (CORE) in Pittsburgh, PA, after standard organ recovery techniques once consent for research use of human tissue was obtained. Islets were isolated using the semi-automated method described by Ricordi et al. (13) with minor modifications (14). The purity of islet preparations was evaluated by dithizone staining (15). The islets were cultured for 1 to 7 days (37°C, 5% CO_2) in CMRL-1066 culture medium (Cellgro Mediatech Herndon, VA) supplemented with 10% heat-inactivated fetal calf serum, 100units/mL penicillin, 0.1mg/mL streptomycin, and 2mmol/L L-glutamine (Life Technologies, Grand Island, NY), until used in the experiments.

Large white crossbred adult sows (n=11) (Wally Whippo, Enon Valley, PA), and adult pigs transgenic for hCD46 (16), a complement-regulatory protein (CRP) (n=3, 2 of which were on a GT-KO background, therefore lacking expression of the Gal epitope) (Revivicor, Blacksburg, VA), were used as pig islet donors. Methods of recovery of pig pancreata, islet isolation and purification, and evaluation of purity and quality have previously been described (17). All pig procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985), and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

BLOOD SAMPLES

Human blood was drawn from healthy volunteers after informed consent, as approved by the University of Pittsburgh Institutional Review Board (IRB#0608179). Human whole blood was drawn into tubes with 1mg/mL ethylenediamine tetraacetic acid (EDTA) for plasma separation or used immediately after being drawn without addition of anticoagulants. Heat-inactivation was carried out by incubation of samples at 56°C effectively for 30 minutes. Pig donor blood was collected into tubes containing EDTA 1mg/mL. EDTA-anticoagulated human donor blood was received together with the donor pancreas and stored overnight until used in experiments. It was recalcified with

calcium chloride (40mM final concentration) to restore its coagulative capacity just before use. Complement activation was only investigated in experiments with freshly-drawn blood.

EXPERIMENTAL DESIGN

Approximately 2,500 human or porcine islet equivalents (IEQ) with a purity of 60-80% were resuspended in 1.0mL of culture medium (CMRL) and placed in 35mm untreated polystyrene Petri dishes (BD Falcon, Franklin Lakes, NJ). One milliliter of freshly drawn human whole blood, or anticoagulated and recalcified donor whole blood, was added in autologous, allogeneic or xenogeneic combinations with the islets. Blood was always ABO blood group-compatible with the islet donor. Plasma alone was added in the amount of 500µL and final volume adjusted accordingly.

Whole blood was added in a 1:1 volume ratio with the islet suspension, which allowed for the sampling of supernatants once the islet-induced fibrinous clot was broken by a pipette tip. This experimental set-up reflects the *in vivo* infusion of islets, resuspended in infusion fluid, into the portal vein. The experiment was performed in an incubator-shaker at 37°C and 100 revolutions per min (rpm). The time until clotting was recorded and compared to controls (1.0mL of blood and 1.0mL of medium, but no islets).

Dishes were set up in quadruplicate to allow for supernatant sampling at 5, 15, 30 and 60min. At sampling, EDTA was added (10mM final concentration) to prevent further complement activation (18). Supernatants were spun and immediately stored at -70°C until further analysis. Samples for analysis of human or porcine C-peptide were stored with 5% aprotinin (Trasylol; Bayer Pharmaceuticals, West Haven, CT) for protein preservation.

In order to better understand the role of coagulation and complement activation in early islet damage, five independent treatments, relevant to xenoTx, were tested. First, LMW-DS (Fluka, Buchs, Switzerland; 1.6mg/mL) was added. LMW-DS has been used *in vitro* to prevent islet-induced coagulation and complement activation (10), and *in vivo* in preclinical islet xenoTx models (12, 19). Second, NAC (an *N*-acetylcysteine derivative, 80mM, kindly provided by Laboratoires SMB, Brussels, Belgium), recently shown to have an effect on downregulating the expression of TF mRNA (20), was tested. NAC additionally has anti-oxidant and anticoagulant effects, possibly due to its direct interference with coagulation factors (20, 21). Third, we tested the effect of hCD46 expressed on pig islets (16, 19). Fourth, we used compstatin, a C3-binding cyclic synthetic peptide that inhibits complement (Tocris Bioscience, Ellisville, MO; 250µmol/L) (22). Fifth, we added anti-IgM antibody (1:100, Kirkegaard & Perry, Gaithersburg, MD). None of these treatments affected islet viability or function, including C-peptide release. Epinephrine was added (1µM in 3 independent experiments) to assess for any possible inhibition of C-peptide release (23).

QUALITATIVE ANALYSIS

Levels of pig and human C-peptide in supernatants were measured by radioimmunoassay (RIA) (Linco Research, St Charles, MO) using species-specific antibodies (without cross-reactivity between human and pig C-peptide). Enzyme-linked immunosorbent assay (ELISA) was used to determine levels of soluble complement activation products: C4d for the classical pathway, Bb for the alternative pathway, and iC3b for the converged complement pathway (all from Quidel Corporation, San Diego, CA). Islet viability was assessed by FACS analysis using propidium iodide (PI) according to the manufacturer's recommendations (BD Bioscience, San Diego, CA). Prior to analysis, islet cells were dissociated in dissociation buffer (Gibco, Carlsbad, CA) pre-warmed at 37°C (24). Islet cells were then incubated for 10min at 37°C while pipetting was carried out every 3-4min to ensure cell dissociation. Islet cells were then washed in PBS containing 0.25% bovine serum albumin (BSA, Fraction V, Sigma, St. Louis, MO) and immediately subjected to FACS analysis (25).

Additionally, cell physiological status was assessed by measuring oxygen consumption by pig islet mitochondria. It was measured by a Clark type oxygen electrode (Oroboros High Resolution Respirometer, Innsbruck, Austria) after a 2h exposure to xenogeneic plasma (or autologous plasma as control) and a 30min glucose starvation. Measurements were performed in Krebs buffer supplemented with 0.5% BSA, 20mM $NaHCO_3$ and 1mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.4. A 2mL water-jacketed chamber was maintained at 37°C and the islet solution was constantly stirred with a magnetic stirring bar. After establishing a stable basal respiration with 5mM glucose, islets were challenged with 20mM glucose, which resulted in the stimulation of oxygen consumption. Addition of oligomycin, which shuts down mitochondrial ATP production, resulted in a sharp decrease in respiration rate. The residual level of respiration is indicative of the leak through the mitochondrial membrane and, in an indirect way, of the effectiveness of ATP production. Finally, to estimate maximal activity of the respiratory chain, islets were challenged with an uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), which dissipates membrane potential and removes all regulatory restrictions from respiratory complexes.

HISTOLOGY

After 60min of incubation, islets cells were fixed in 2% paraformaldehyde, and frozen for immunofluorescent staining. Cryo-sections were cut and stained using standard immunofluorescent procedures. The primary antibodies were

goat anti-human IgG and IgM (1:1000, Kirkegaard&Perry), rabbit anti-human C4d (1:20, EMELCA Bioscience, Bergen op Zoom, The Netherlands), mouse anti-human C5b-9 (1:100, Abcam, Cambridge, MA), and rabbit or mouse anti-insulin (1:100, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were goat anti-mouse Cy3, goat anti-rabbit Cy3, donkey anti-goat Cy3 (1:500, all from Jackson ImmunoResearch, West Grove, PA), donkey anti-rabbit Alexa 488 and goat anti-rabbit or mouse Alexa 488 (1:500, Molecular Probes, Eugene, OR).

Photographs were taken through a Nikon Eclipse E800 microscope with a Photometrics Cool SNAP digital camera and Nikon C1 confocal system at 40x objective lens and analyzed by MetaMorph imaging analysis software (Molecular Devices, Downingtown, PA).

For each condition, multiple images from at least 2 independent experiments were taken and analyzed. Representative images were selected.

STATISTICAL ANALYSIS

Continuous variables are expressed as mean±SEM, and compared using the Student t-test. Linear regression was used to analyze if increases over time in supernatant products were significant, and differences in slope were compared. p-values <0.05 were considered to indicate a statistically significant difference. All analyses were performed with GraphPad Prism 4 for Macintosh (GraphPad Software, La Jolla, CA).

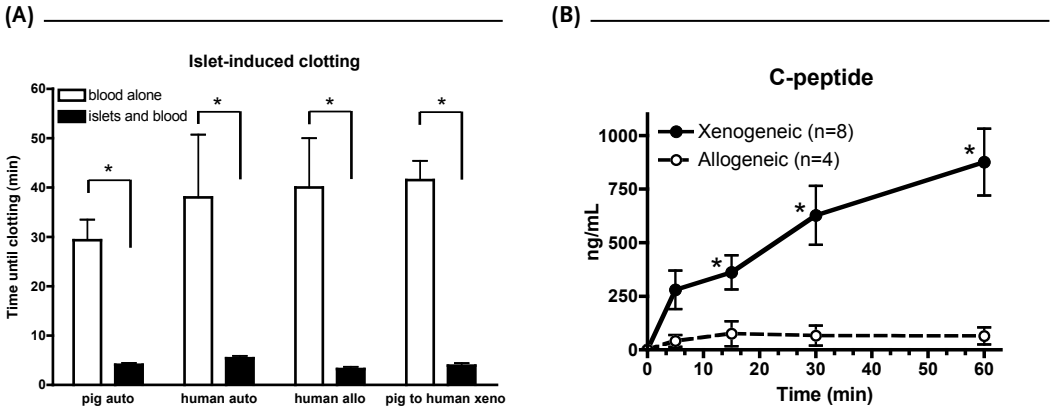

FIGURE 1: (A) Islets induced complete clotting within 6min when incubated with whole blood, regardless whether autoTx, alloTx or xenoTx was modeled. Data are mean±SEM of at least 4 independent experiments. *p<0.01. (B) The release of C-peptide, as a measure of beta cell damage, was significantly higher in xenogeneic (pig islets-human blood) than in allogeneic (human islets-human blood) combinations. *p <0.05.

Results

ISLET-INDUCED CLOTTING OF WHOLE BLOOD

In this *in vitro* test, clotting of freshly-drawn, non-anticoagulated human blood, and of pig or human blood that was EDTA-anticoagulated and subsequently recalcified, occurred in 30-40min on average and did not significantly differ (p>0.05). Addition of islets to the blood rapidly induced total clotting in human autologous (5:26±0:29min, p<0.01 vs. control, thus blood only), pig autologous (4:06±0:21min, p<0.001 vs. control), human allogeneic (3:13±0:27min, p<0.001 vs. control), and pig-to-human xenogeneic combinations (3:54±0:32min, p<0.001 vs. control) (Figure 1A). Clotting time did not significantly differ when comparing autologous, allogeneic, and xenogeneic settings (p>0.05).

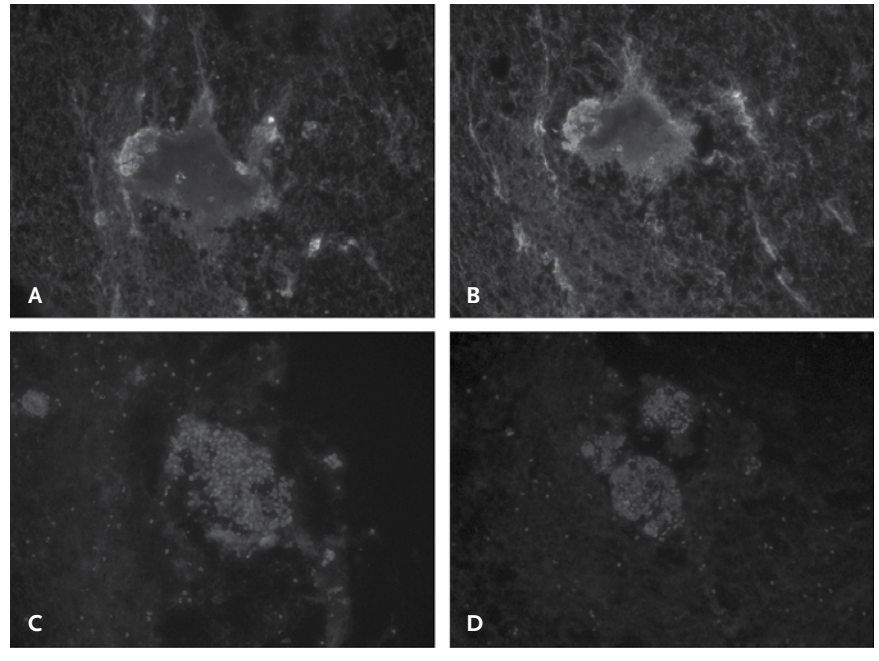

FIGURE 2: Binding of human IgM and IgG antibody to pig islets (xenogeneic) (A-B), and to human islets (allogeneic) (C-D). IgM (green, A,C), IgG (green, B,D), insulin (red), nucleus (DAPI/blue). Yellow indicates co-localization of insulin and IgM/IgG. (Color figure in appendix)

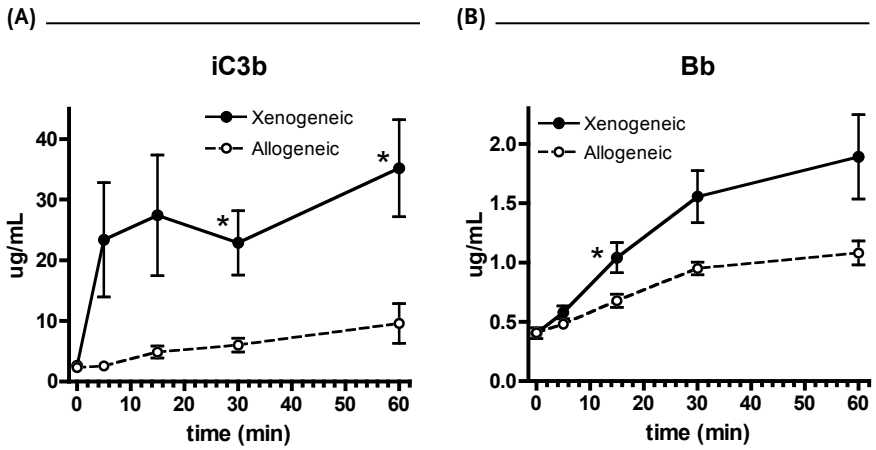

FIGURE 3: (A) At 30 and 60min, pig islets (xenogeneic, n=8) had induced significantly higher levels of the complement activation product iC3b in the fluid phase (i.e., in supernatant), compared with human islets (allogeneic, n=5). (B) Supernatant levels of Bb induced by pig islets (xenogeneic, n=8) and human islets (allogeneic, n=5). *p<0.05.

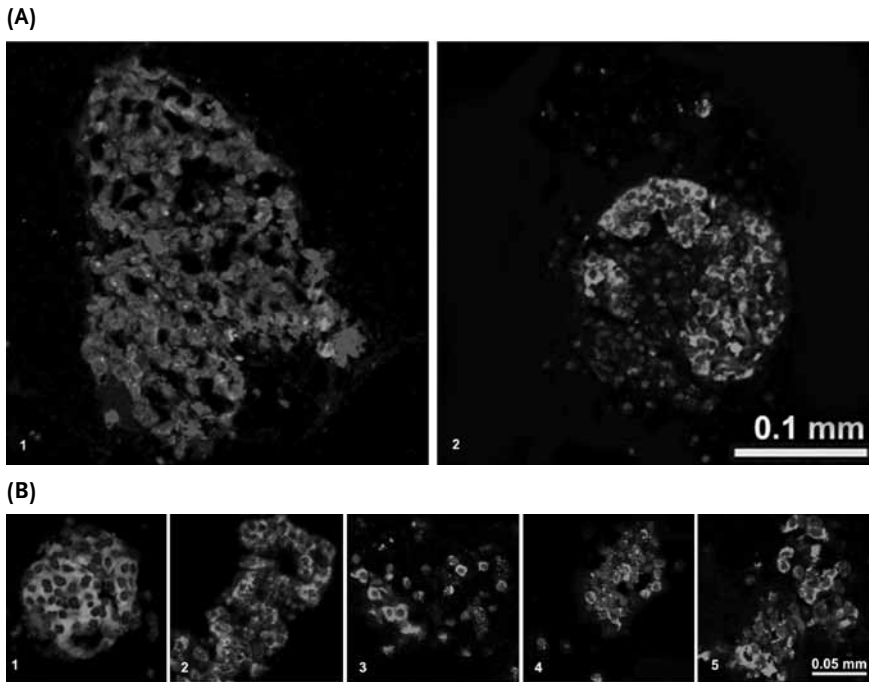

FIGURE 4: (A) Greater immunofluorescent C5b-9 (membrane-attack complex) positivity was detected on pig islets exposed to human blood (1) when compared to human islets in contact with human blood (2). C5b-9 (red), insulin (green), nucleus (DAPI/blue). (B) C5b-9 staining on pig islets after treatment with: LMW-DS (1), NAC (2), compstatin (3) and anti-IgM (4). hCD46 transgenic pig islet (5). C5b-9 (red), insulin (green), nucleus (DAPI/blue). (Color figure in appendix)

C-PEPTIDE RELEASE AS AN INDICATOR OF BETA CELL DAMAGE

Figure 1B shows the release of C-peptide during the 60min *in vitro* experiments, comparing allogeneic vs. xenogeneic islet-to-blood combinations. Exposure of pig islets to human blood caused a significant increase in release of pig C-peptide to 876±156ng/mL (linear regression $p<0.0001$, $r^2=0.48$). In contrast, an increase of C-peptide from human islets was not observed (linear regression $p>0.05$, $r^2=0.05$). The release of C-peptide by pig islets was significantly greater than by human islets, as evidenced by a difference in slope ($p<0.001$) and higher mean values at 15, 30 and 60 minutes (Figure 1B). The addition of epinephrine failed to decrease the release of C-peptide (data not shown), further indicating that C-peptide release was due to a membrane leakage rather than to a physiologic response.

BINDING OF IgM AND IgG ANTIBODIES AND ACTIVATION OF COMPLEMENT

Cryo-sections of islets embedded in blood clots were fixed after 60min and analyzed for antibody binding by immunofluorescence. IgM and IgG antibody staining was observed on pig islets incubated with human blood (Figure 2A-B). No difference in this respect was observed when WT pig islets were compared with GT-KO islets (not shown). In contrast, antibody binding was virtually absent on human islets incubated with allogeneic human blood (Figure 2C-D).

Complement activation, possibly subsequent to antibody binding, was assessed by measuring the soluble complement fragments iC3b, C4d and Bb as markers of activated common, classical and alternative complement pathways, respectively. Figure 3 compares the increments of iC3b and Bb in xenogeneic (pig islets-human blood) and allogeneic (human islets-human blood) experiments. Human islets significantly induced low levels of iC3b (linear regression $p=0.001$, $r^2=0.98$). Pig islets induced significantly higher levels of iC3b at 30 and 60min (Figure 3A). However, the slope of its regression line did not reach significance (linear regression $p=0.144$, $r^2=0.56$), likely due to variability between various samples. Human islets caused a low-grade increase in Bb over time (linear regression $p=0.013$, $r^2=0.91$). The increase caused by pig islets was also significant (linear regression $p=0.011$, $r^2=0.91$), and significantly greater than the one caused by human islets ($p=0.033$). Mean levels differed at 15min (Figure 3B). No changes in C4d levels (fluid phase) were observed under any of the experimental conditions, and immunofluorescent staining for C4d (solid phase) remained negative (data not shown).

C5b-9, the membrane-attack complex (MAC) that causes cell lysis, was more abundant on pig islets exposed to human blood than on human islets exposed to human blood (Figure 4A, 1-2).

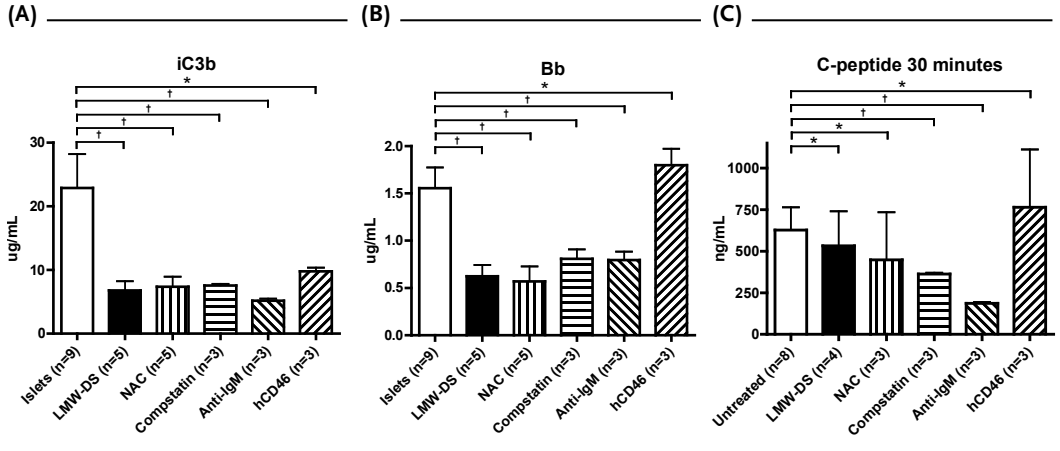

FIGURE 5: Levels of fluid phase (i.e., in supernatant) iC3b (A) and Bb (B), induced by pig islets exposed to blood for 30 minutes. Untreated vs. LMW-DS, NAC, compstatin, anti-IgM, and hCD46 expression, respectively. All the therapeutic models, except hCD46 expression, significantly reduced complement activation. (C) C-peptide release after 30 minutes contact of pig islets with human blood. Compstatin and anti-IgM efficiently prevented C-peptide release, indicating a reduction in cell damage. *p>0.05. †p<0.05.

EFFECTS OF MODULATING TREATMENTS IN XENOGENEIC BLOOD-ISLET COMBINATIONS

As Figure 5A-B shows, all therapeutic modalities, except the transgenic expression of hCD46 on the islets, significantly reduced iC3b and Bb levels. Lack of, or partial inhibition by hCD46 could be related to its complement-regulatory effect that occurs at a later stage in the complement cascade. Whereas the addition of LMW-DS attenuated but did not prevent C5b-9 deposition (Figure 4B, 1), C5b-9 was not found after NAC, compstatin or anti-IgM treatment (Figure 4B, 2, 3, 4), nor on hCD46 transgenic islets (Figure 4B, 5). Porcine C-peptide released after exposure of pig islets to blood (Figure 5C) was significantly reduced by addition of compstatin and anti-IgM, but not significantly altered by LMW-DS, NAC or hCD46 expression. The anticoagulant effect, in itself, by LMW-DS, therefore, did not prevent islet damage.

EFFECT OF EXPOSURE OF PIG ISLETS TO HUMAN PLASMA

Evidence that complement and antibody modulation resulted in reduced islet cell leakage, led us to focus on humoral blood components and their possible involvement in early islet loss. Figure 6A shows that C-peptide concentrations, measured in the supernatant of pig islets exposed to human plasma, were comparable to those after exposure to whole blood. The lack of difference between whole blood and plasma suggests that early damage occurred as effects of humoral factors, which heat-inactivation efficiently modulated (Figure 6A).

To further assess the extent of the damage, we quantified the number of PI-positive (i.e., dead) cells, comparing experimental (islets and plasma, islets and heat-inactivated plasma) with control conditions (islets and autologous plasma). Data showed (Figure 6B) a statistically significant increase in PI-positive cells in plasma-exposed islets, while heat-inactivation allowed for some protection (though statistically non-significant).

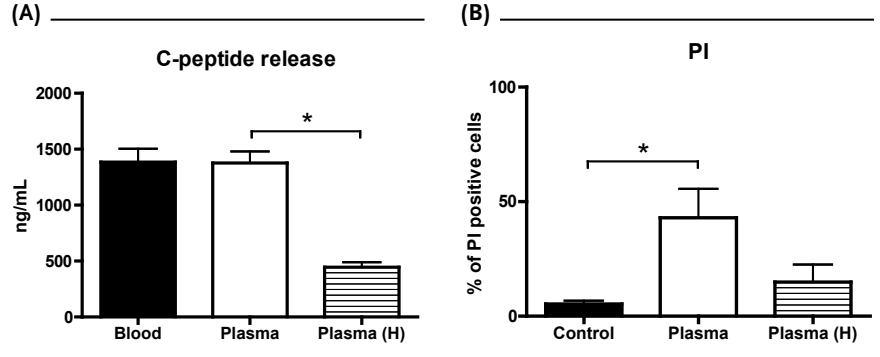

FIGURE 6: (A) C-peptide release after contact of pig islets with human blood, plasma or heat-inactivated plasma (H). Exposure of pig islets to human blood was compared to human plasma and human heat-inactivated plasma. The difference between plasma and heat-inactivated plasma was statistically significant. *p<0.05. (B) Percentage of propidium iodide (PI)-positive (i.e., dead) islet cells after exposure to plasma or heat-inactivated plasma (H). Pig islets exposed to autologous plasma were used as control. Control vs. plasma. *p<0.05.

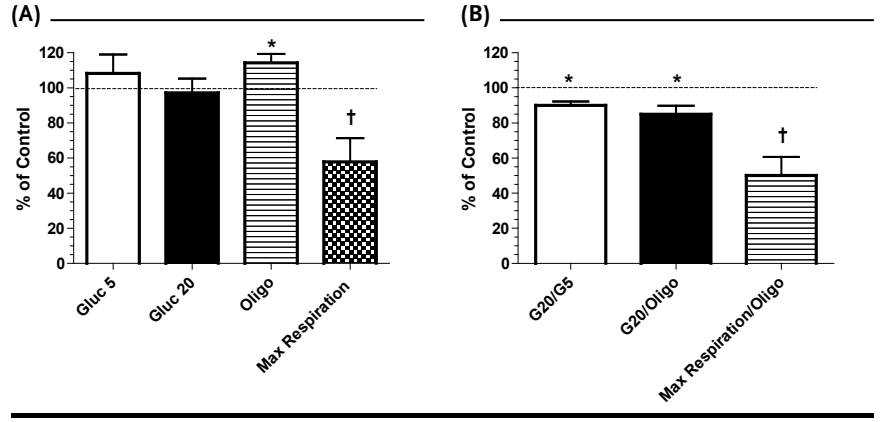

FIGURE 7: (A) Changes in oxygen consumption rates of pig islets exposed to xenogeneic plasma expressed as % of that of pig islets exposed to autologous plasma (set at 100%). At 5mM glucose (Gluc5) and after exposure to oligomycin (Oligo) oxygen comsumption was increased, indicating increased leakage through the mitochondrial membrane, consistent with cellular injury. The strongly reduced maximum respiration (Max Respiration) is conform this observation (B) Ratios of respiratory parameters for plasma-treated islets (% of control). Islets exposed to xenogeneic plasma demonstrated a smaller response to high glucose as judged by the ratio of respiration in the presence of 20mM glucose to basal respiration (G20/G5). They also had a smaller G20/Oligo ratio, and a dramatic decrease in respiratory control (Max respiration/Oligo). These observations indicate impairment of insulin release and mitochondrial functions in these cells. *p<0.05 and †p<0.001 compared to control.

As Figure 7A shows, pig islets exposed to xenogeneic plasma had elevated basal mitochondrial respiration as well as respiration in the presence of oligomycin, when compared to autologous plasma, indicating increased leakage through the mitochondrial membrane. Increased membrane leakage translates into less production of ATP for cellular energetic needs. Exposure to xenogeneic plasma also caused a striking effect on the maximal respiratory activity of islet mitochondria by decreasing it twofold. All these observations are symptomatic of mitochondrial machinery dysfunction and are consistent with cell injury and islet apoptosis.

Plasma exposure was also associated with deposition of C5b-9 on islets (C5b-9 positivity), as well as IgM binding (Figure 8A-B). Deposition of C5b-9 and IgM were reduced if the plasma was heat-inactivated (Figure 8C-D).

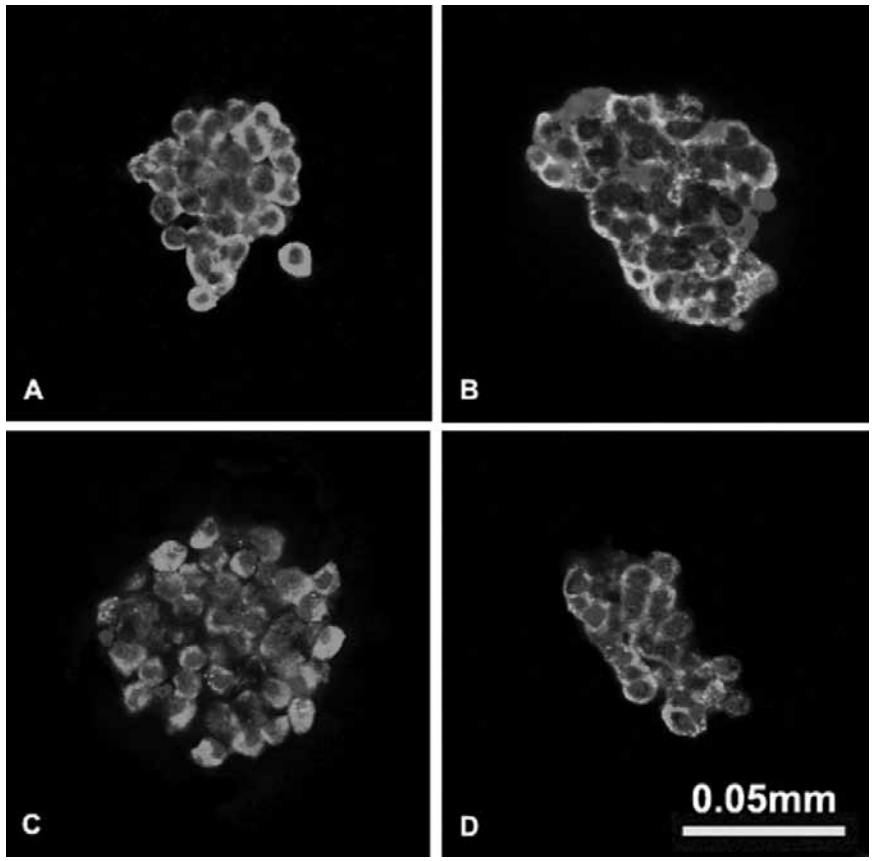

FIGURE 8: C5b-9 and IgM deposition after exposure of pig islets to human plasma (A-B) and to heat-inactivated plasma (C-D). C5b-9 (red, A and C), IgM (red, B and D), insulin (green), nucleus (DAPI/blue). (Color figure in appendix)

Discussion

The present study was aimed at characterizing the pathophysiological events triggered during direct exposure of pancreatic islets to blood, as occurs after islet Tx into the portal vein. Incubating pig and human islets with human blood proved a valuable model to investigate the events, as previously shown in a similar model by Dwyer et al. (26). A non-physiologic C-peptide release was observed *in vitro*, indicating acute beta cell damage, confirming similar *in vivo* reports after islet alloTx and xenoTx (8, 9). The release of C-peptide from pig islets exposed to human blood was significantly greater than from equal numbers of human islets, indicating a greater lysis of the islets, which suggests that species incompatibility plays a pivotal role.

Despite this difference in C-peptide release, activation of blood coagulation occurred equally rapidly in allogeneic and xenogeneic combinations. Even when autologous combinations were modeled, clotting occurred equally rapidly. However, anticoagulation did not prevent islet leakage, which correlates with previous reports that anticoagulation alone is insufficient to inhibit IBMIR and early islet loss (27, 28). Nevertheless, anticoagulation to prevent portal vein thrombosis during islet intraportal infusion is certainly necessary.

Our next objective was to better understand the role of complement, which has previously been demonstrated to play a central role in IBMIR (6). In this *in vitro* model, levels of iC3b, an indicator of the early terminal complement pathway, were significantly increased when pig islets were exposed to whole human blood. The complement cascades led to the formation of the cytolytic product (MAC or C5b-9). Pig islets exposed to human blood stained clearly positive for C5b-9. In contrast, human islets did not cause a significant increase of iC3b and, concomitantly, the extent of C5b-9 immunostaining was less.

Activation of the complement system can occur through several pathways, of which the classical and alternative pathways may be the most important (29). Eventually both pathways lead to high iC3b and MAC formation. In xenogeneic combinations (i.e., pig islets with human blood), we evidenced involvement of both activation pathways. Pig islets exposed to human blood were positively immunostained for both IgM and IgG, indicative of the classical pathway. The use of GT-KO pigs (in association with the hCD46 transgene) did not show any

protective effect, suggesting that antibody binding to non-Gal antigens would likely be a potent complement activator. This correlates with the observation that natural anti-Gal antibodies do not appear to be detrimental to survival of adult pig islets (30-33) due to the low expression of Gal on these islets (12, 34). Using a large particle flowcytometric technique, others also reported deposition of IgM and IgG antibodies on human and pig islets in *in vitro* models of IBMIR (35,36).

Activation of the alternative pathway depends on the subtle balance between spontaneously-deposited low levels of C3b and exposure of cell surface complement-regulatory molecules (37). When regulation of activation fails (as can be postulated when human complement binds to the pig cell surface, due to incompatibilities between human complement and pig complement-regulatory molecules), deposited C3b binds factor B producing Bb. With the use of pig islets expressing hCD46, an attempt was made to inhibit the complement cascade. CD46 is a complement-regulatory protein that is able to protect the host cell against complement injury by modulating proteolytic cleavage of C4b and C3b (38). Its features were maintained when transgenically-expressed on pig cells (16). hCD46 acts to restrict complement activation mediated by the alternative pathway on the cell surface, rather than in the fluid phase (38). Indeed, in the current experiments hCD46 expression on pig islets did not reduce fluid phase levels of Bb, but appeared to reduce C5b-9 deposition on the islet cell surface. However, no clear protective effect regarding C-peptide release was observed.

In addition to direct membrane damage of beta cells, the inflammatory response is likely to induce a significant degree of apoptosis (39). Although this could be investigated in our *in vitro* model, it would be difficult to monitor *in vivo* after intraportal islet Tx. In contrast, C-peptide levels can be of immediate use after islet Tx as a measure of islet damage.

Our *in vitro* model was also intended to enable testing compounds that could exert a protective effect on the islets. Blockade of coagulation and complement by LMW-DS, NAC and hCD46 were insufficient to fully prevent the release of C-peptide by pig islets exposed to human blood. Only compstatin and neutralizing anti-IgM antibodies modulated complement activation and prevented islet damage.

The need for efficient complement inhibition (e.g., with compstatin) and prevention of IgM binding to achieve islet protection, indicated a relevant role for humoral factors. The experiments using human plasma further stressed this role. Xenogeneic human plasma triggered the same porcine C-peptide release as whole blood. Furthermore, plasma was sufficient to induce impairment of cell respiration and islet cell death. Mitochondrial dysfunction is likely among

the mechanisms of cell death as a result of cell-plasma interaction. The effect of plasma was efficiently prevented by heat-inactivation. These observations strongly suggest that, in the pig-to-primate model, antibody binding and activation of the classical complement cascade may be playing a much more important role in early islet loss than previously considered.

Several strategies to prevent early islet loss are currently being investigated. Some aim at altering the interface between the islet surface and host environment, i.e., by covering the islets with a heparin or polyethylene glycol coating (28, 40), or by alginate encapsulation (41). This might provide a barrier for antibody binding and complement activation. Pigs with multiple genetic modifications, including expression of complement-regulatory and/or 'anti-thrombotic' transgenes, as an alternative islet-source may prove beneficial in reducing islet cell loss from IBMIR (19, 26, 42). Compstatin may soon be a clinically-applicable complement inhibitor (43). If the important role of xenoreactive antibodies is confirmed in further (*in vivo*) experiments, pre-Tx antibody-neutralizing therapies (e.g., immunoadsorption, plasmapheresis, IVIg) may be investigated in NHP models of islet xenoTx. Finally, choosing a non-intravasular site, different from the portal vein, may be necessary (44). Our group has shown that IBMIR can be prevented in a pig alloTx model if islets are transplanted into the gastric submucosal space, a site where direct contact with the blood stream is avoided, but with a rich arterial blood supply for delivery of oxygen and nutrients (45).

Nonetheless, our data point to plasmatic factors as central players above and beyond pro-coagulative events and blood cellular interplay. Humoral immunity should be efficiently downregulated to prevent early islet loss and to make islet alloTx and, particularly, xenoTx a safe and successful form of therapy.

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TRANSGENIC ISLETS

CHAPTER 4

Long-term controlled normoglycemia in diabetic non-human primates after transplantation with hCD46 transgenic porcine islets

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ABSTRACT

XenoTx of porcine islets into diabetic NHPs is characterized by (i) an initial massive graft loss possibly due to the IBMIR, and (ii) the requirement of intensive, clinically-unfriendly immunosuppressive therapy. We investigated whether the transgenic expression of a human complement-regulatory protein (hCD46) on porcine islets would improve the outcome of islet xenoTx in streptozotocin-induced diabetic cynomolgus monkeys. Immunosuppression consisted of thymoglobulin, anti-CD154 mAb for costimulation blockade, and mycophenolate mofetil. Following the Tx of islets from WT pigs (n=2) or from GT-KO pigs (n=2), islets survived for a maximum of only 46 days, as evidenced by return to hyperglycemia and the need for exogenous insulin therapy. The Tx of islets from hCD46 pigs resulted in graft survival and insulin-independent normoglycemia in four of five monkeys for the 3 months follow-up of the experiment. One normalized recipient, selected at random, was followed for >12 months. Inhibition of complement activation by the expression of hCD46 on the pig islets did not substantially reduce the initial loss of islet mass, rather was effective in limiting antibody-mediated rejection. This resulted in a reduced need for immunosuppression to preserve a sufficient islet mass to maintain normoglycemia long-term.

Introduction

Excellent short-term results with islet alloTx have been achieved (1). However, the shortage of donor islets, poor long-term outcomes (2) and the risk of allosensitization (3), jeopardizing future kidney Tx in type 1 diabetic patients, have led to active discussions about future directions of this field (4-7). The need for data indicating the efficacy of alternative islet sources in NHP models has been highlighted (4). Successful xenoTx of porcine insulin-producing cells into diabetic patients could restore physiological islet function, without the risk of allosensitization (8). Pig insulin has been successfully used for years in treating diabetic patients. Long-term pig islet survival under a limited immunosuppressive protocol would significantly expand the clinical applicability of beta cell replacement therapy for diabetes.

Previously, two groups demonstrated the feasibility of pig islet Tx in NHP (9, 10). One group achieved function of adult islets using a multi-drug immunosuppressive protocol that was not considered clinically-applicable (9). The other group reached a significant, but poorer, degree of metabolic control using neonatal pig islets and less immunosuppression (10). These results indicate that xenoTx can become a reliable treatment option for diabetes only if new strategies are developed that overcome the xeno-immunologic hurdles and avoid the use of intensive immunosuppression.

Binding of natural and induced antibodies, and activation of complement are part of the xenograft rejection mechanism (11). In addition, complement activation is a key effector mechanism of the IBMIR, a nonspecific inflammatory phenomenon occurring immediately after islet Tx into the portal vein that prevents a major proportion of infused islets from engrafting (12, 13).

We hypothesized that the use of islets from pigs Tg for human CD46 (hCD46), a human CRP, would allow more islets to survive the IBMIR and complement-mediated rejection, thus more efficiently restoring normoglycemia in cynomolgus monkeys with chemically-induced diabetes. Herein we demonstrate that, although the immediate loss of a significant proportion of transplanted islets could not be prevented, engrafted hCD46 porcine islets can actually function long-term. Consequently, the applied immunosuppression did not lead to drug-related morbidity and mortality in monkey recipients, advancing the field of islet xenoTx toward clinical application.

Materials & Methods

SOURCES OF ANIMALS

Two WT female outbred Large White pigs (Wally Whippo, Enon Valley, PA), two GT-KO female pigs (pigs free from Gal expression) (Revivicor, Blacksburg, VA), and seven hCD46 female pigs aged 7 months to 2 years (Revivicor), all weighing >180kg, were used as islet donors. Nine male cynomolgus monkeys (*Macaca fascicularis*; Three Springs Scientific, Perkasie, PA), 2-5 years of age and weighing 3.7±0.5 kg were used as islet recipients.

All animal care procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985), and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

PRODUCTION OF hCD46 TRANSGENIC PIGS

The hCD46 pigs that were sources of islets were F4 and F5 progeny derived by outcrossing from a hCD46 progenitor line previously described (14). The hCD46 transgene, a minigene under control of its endogenous promoter, was optimized for high ubiquitous expression of this CRP, as demonstrated by robust immunohistochemistry staining in all tissues analyzed, including all cell types observed in fetal, neonatal, and adult pancreas tissues (14), and isolated adult islets (Figure 1B-C).

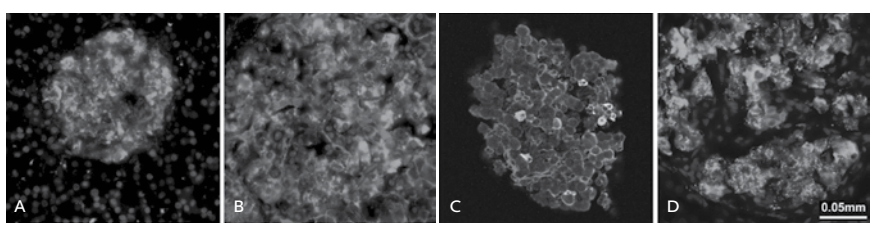

FIGURE 1 | EXPRESSION OF hCD46: Anti-insulin (green) and anti-hCD46 (red) antibody staining of pig pancreatic tissue and islets. (A) WT pig pancreas (Group A); (B) hCD46 transgenic pig pancreas (Group B). (C) Isolated hCD46 transgenic pig pancreatic islet prior to transplantation; and (D) islet in the liver of the monkey recipient one year after transplantation (Group B). (Color figure in appendix)

IVGTT AND AST BEFORE AND AFTER DIABETES INDUCTION, AND AFTER TRANSPLANTATION

For blood withdrawal and i.v. drug administration in the monkeys, catheters were inserted into the carotid artery, jugular vein and/or the stomach, and connected through a tether and jacket system to the exterior of the animal cage.

Monkeys underwent intravenous glucose tolerance tests (IVGTT) and arginine stimulation tests (AST) before induction of diabetes, as previously described (15). The acute C-peptide response after glucose stimulus (ACR_{Glu}) was calculated as the mean of post-challenge C-peptide values obtained at 5 and 15min minus the corresponding pre-challenge value. The response after arginine (ACR_{Arg}) was calculated as the difference between the C-peptide value at 2min and the baseline value.

Diabetes was induced by the i.v. injection of streptozotocin (STZ, Zanosar, 125-150mg/kg, but not exceeding 1500mg/m^2 to avoid nephrotoxicity; Sicor Pharmaceuticals, Irvine, CA). Monkeys were considered diabetic if (i) they had persistent hyperglycemia (>350mg/dL on at least two occasions, measured twice daily using Freestyle; Abbott Laboratories, Abbott Park, IL), (ii) they required exogenous insulin administration (HumulinR; Eli Lilly, Indianapolis, IN) to prevent ketoacidosis, (iii) baseline primate C-peptide, determined by RIA (Linco Research, St Charles, MO), was reduced by >75% after STZ (16), and importantly non-responsive to IVGTT and AST. If these criteria were not met, even after a second dose of STZ, monkeys were excluded from the study.

IVGTT and AST were also carried out at various time points after Tx to document graft metabolic performance.

ISLET ISOLATION AND ISLET QUALITY ASSURANCE

Pig pancreata were recovered during a non-survival surgical procedure after *in situ* perfusion with cold Hank's Balanced Salt Solution (HBSS) and avoidance of warm ischemia. Organs were transported immediately to the laboratory to begin the isolation procedure. Porcine islets were isolated, purified, and cultured as previously reported (17). Following isolation, islets were counted and the number expressed as IEQ (17). On the day of islet Tx, after overnight culture, viability and purity were assessed as previously described (17).

For *in vitro* functional studies, islet preparations were subjected to dynamic secretagogue challenges with glucose and theophylline (17). Eluates were collected every minute for measurement of insulin concentration for calculation of a stimulation index (SI) (Table 1). SI indicates the ratio of insulin release induced by high glucose (plus theophylline) over insulin release induced by low glucose.

Table 1 Recipient groups, donor and islet characteristics, immunosuppressive drug exposure.

Recipient ID	Donor pig	IEQ/kg	Viability (%)	Purity (%)	Stimulation index (high gl/high gl+theoph)	CD3 Tcells on day of Tx (cells/μL)	Weekly MMF trough level (μg/mL)	Weekly Anti-CD154 trough level (μg/mL)
Group A								
M84-05	WT	100,000	90	90	2.4/6.3	75	ND	641
M1-06	GT-KO	85,000	95	85	2.2/8.1	518	3.93±1.18	1138±440
M2-06	WT	100,000	85	65	2.6/6.2	315	3.11±2.31	1021±489
M4-06	GT-KO	100,000	90	80	4.0/6.1	36	4.58±1.44	1388±578
Group B								
M29166	hCD46	90,000	87	90	3.9/5.5	5	2.10±1.27	1075±514
M122-08	hCD46	95,000	85	80	2.1/8.7	338	3.31±1.39	609±165
	hCD46*	100,000	90	85	2.3/4.8	37		
M6075	hCD46†	94,000	95	90	2.3/16.0	608	3.22±1.41	944±461
M174-08	hCD46	100,000	90	90	ND	293	4.22±1.92	663±184
	hCD46‡	100,000	90	85	3.0/4.7	23		
M7273	hCD46	100,000	87	90	3.9/5.5	398	2.64±1.87	744±415

*IEQ = Islet equivalent; high gl/ high gl+theoph = high glucose/high glucose + theophylline; Tx = Transplantation; MMF = Mycophenolate mofetil; WT = Wild-type; GT-KO = α1,3-galactosyltransferase gene-knockout; ND = Not determined; * re-Tx on day 49; † Donor pig was progeny of hCD46 pig crossed with WT pig and was heterozygous for hCD46. All other pigs were homozygous for hCD46; ‡ re-Tx on day 91*

ISLET TRANSPLANTATION AND RECIPIENT GROUPS

Islets were resuspended in 20mL CMRL-1066 medium supplemented with LMW-DS (4.5mg/kg of recipient body weight) for anticoagulation. Under full anesthesia and after laparotomy, Tx was carried out by infusion of the islets into the portal vein by gravity over a period of 5-10min. Peri-operative care consisted of cefazolin for antibiotic and buprenorphine for analgesic treatment.

Monkey islet recipients were divided into two groups. Group A recipients (n=4) were transplanted with either WT porcine islets (n=2) or islets isolated from GT-KO pigs (n=2), in numbers of 85,000 to 100,000 IEQ/kg body weight. Group B recipients (n=5) received equal numbers of islets from hCD46 pigs. All monkeys were transplanted with islets from a single pig donor. Two Group B monkeys were retransplanted after 49 and 91 days, respectively (Table 1). Table 2 summarizes some additional information on the monkey recipients.

IMMUNOSUPPRESSION

Immunosuppressive therapy was identical for both groups (Figure 2). Induction therapy consisted of 25mg anti-thymocyte globulin i.v. (ATG, Thymoglobulin; Genzyme, Cambridge, MA) on day -3, followed by 5-25mg on day -1, depending on efficacy of the initial dose, and aiming for $CD3^+$ T cell numbers of <500cells/μL whole blood on the day of islet Tx, determined by flow cytometry. One monkey was treated with a single dose of 75mg ATG on day -1. Immunosuppression was maintained by the oral administration of mycophenolate mofetil (MMF, Cellcept, 50-100mg/kg/day to obtain trough levels of 3-5μg/mL; Roche Pharmaceuticals, Nutley, NJ), and i.v. injections of anti-CD154 mAb (ABI793, 25mg/kg on days -1, 0, 3, 7, 11 and 15; generously provided by Novartis Pharma, Basel, Switzerland). Anti-CD154 is a humanized mAb cross-reactive with NHP CD154 that inhibits the costimulatory signal between CD40 on the antigen presenting cell and CD154 on the T helper cell (18). After 15 days, the dose was reduced to weekly injections of 5-15mg/kg to maintain trough levels of 500μg/mL, as measured by quantitative ELISA (19).

We used an anticoagulant, anti-inflammatory treatment protocol developed based on our previous experiences with IBMIR (20), aiming at limiting its detrimental effects during the peri-Tx period (Figure 2). Fifteen minutes before Tx, a bolus of 10mg/kg methylprednisolone (Solu-Medrol; Pfizer, New York, NY)

Table 2 Recipient characteristics.

	Body Weight Pre STZ/Tx/End	STZ dose (mg/Kg)	Time between STZ and Tx (days)	Insulin requirements prior to Tx IU/24hr (IV)	Tx Follow-up (days)
Group A					
M84-05	3.6/3.6/3.6	150	15	1.76	8
M1-06	4.2/3.9/4.0	150	45	3.12	27
M2-06	3.5/3.2/2.8	150	15	3.04	50
M4-06	4.2/3.9/3.5	125	16	4.4	37
Group B					
M29166	3.1/3.0/3.2	150	15	2.24	92
M122-08	3.0/2.8/2.9	125x2 doses	17 1st Tx 66 2nd Tx	3.89 pre-Tx (1st) 1.60 pre-Tx (2nd)	91 from 2nd Tx
M6075	4.5/4.3/5.0	150	66	11 SQ	92
M174-08	3.9/3.7//4.4	125	20 1st Tx 91 2nd Tx	4.88 pre-Tx (1st) 2.4 pre-Tx (2nd)	90 from 2nd Tx
M7273	3.2/3.0/4.9	150	16	6.56	396

SQ = subcutaneous

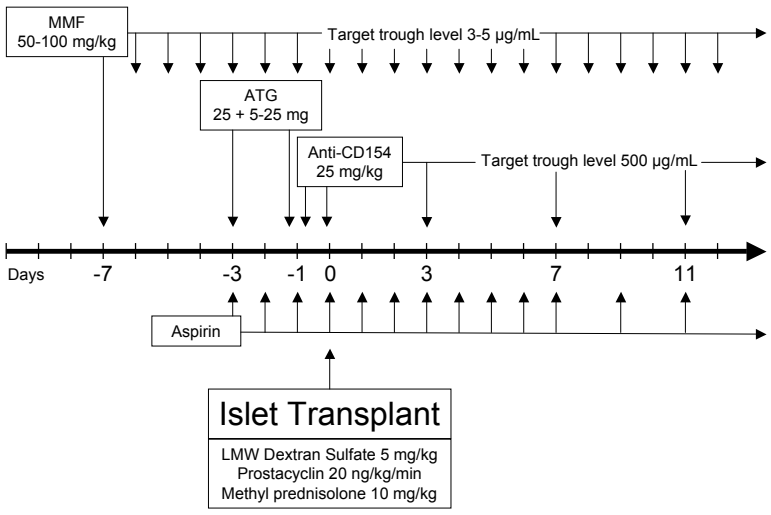

FIGURE 2 | EXPERIMENTAL DESIGN: After 14 days, the administration of weekly injections of 5-15 mg/kg anti-CD154 was sufficient to maintain trough levels >500 µg/mL.

to reduce inflammation, and a bolus of 5mg/kg LMW-DS for its anticoagulation and anti-complement effects (21), were infused. Subsequently, LMW-DS administration was continued at 2mg/h for 6h. However, in some cases it was stopped earlier, based on clinical signs of increased bleeding risks (persistent oozing of the laparotomy wound). Prostacyclin (Flolan; GlaxoSmithKline, Philadelphia, PA) was administered i.v. at 20ng/kg/min beginning 30min before islet Tx and continued for 6h for its suppressive effect on endothelial cell activation. From day -7 until 7, monkeys were given aspirin, 81mg daily, for reduction of both inflammation and platelet aggregation. After 7 days, aspirin dosage was reduced to 81mg every other day to maintain the anti-platelet effects to prevent the potential thrombotic adverse effects associated with anti-CD154 therapy.

When immunosuppression was started, antiviral prophylaxis consisted of ganciclovir (Cytovene; Roche), administered i.v. at 5mg/kg/day, and orally at 25mg/kg twice daily after vascular catheters were removed. Famotidine was given i.v. or orally at 0.25mg/kg/day for prevention of gastric stress ulceration.

MEASURES OF OUTCOME

Monkeys were followed for 3 months or until graft failure occurred. Functional islet survival was defined as the time after Tx during which fasting porcine C-peptide was detectable, and exogenous insulin requirements were reduced to <50% of pre-Tx levels in order to maintain blood glucose levels <200mg/dL. Porcine C-peptide can be readily distinguished from primate C-peptide using a porcine-specific RIA (Linco Research). Graft failure was determined by exogenous insulin needs exceeding 50% of baseline for more than 2 consecutive days. In addition, days of complete insulin-independence were recorded.

HISTOLOGY

At necropsy, sections of the liver and the native pancreas were fixed in 4% paraformaldehyde and frozen. Standard immunofluorescent procedures were applied to cut sections and capture images for qualitative analysis. The primary antibodies were mouse anti-human CD46 (1:100, Thermo Fisher Scientific, Fremont CA), rabbit anti-human C4d (1:20, EMELCA Bioscience, Bergen op Zoom, The Netherlands), goat anti-human IgG and IgM (1:1000, Kirkegaard & Perry, Gaithersburg, MD), mouse anti-macrophage (1:200, Millipore Corporate, Billerica, MA), rabbit anti-CD97 (1:200, Thermo Scientific, Rockford, IL) rabbit or mouse anti-insulin (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-glucagon (1:50, Zymed, San Francisco, CA) Secondary antibodies: goat anti-mouse Cy3, goat anti-rabbit Cy3, donkey anti-goat Cy3 (1:500, Jackson ImmunoResearch, West Grove, PA), donkey anti-rabbit Alexa 488 and goat anti-rabbit or mouse Alexa 488 (1:500, Molecular Probes, Eugene, OR).

Additionally, liver tissue was fixed in 10% buffered formalin, and sections were stained with hematoxylin and eosin (H&E) and immunoreactive pro-insulin antibodies (Scytek Laboratories, Logan, UT) using standard procedures.

Photographs were taken through a Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY). Images were captured by a Photometrics Cool SNAP digital camera (Roper Scientific, Tucson, AZ) and Nikon C1 confocal system at 40x objective lens and analyzed by MetaMorph imaging analysis software (Molecular Devices, Downingtown, PA).

XENOREACTIVE ANTIBODIES

Recipient sera were tested for binding of xenoreactive antibodies to pig cells before Tx and after 1 week, 1 month and at the end of the study according to methods previously described (22). Briefly, 10^5 WT or GT-KO pig target cells were incubated with 5% heat-inactivated recipient serum or FACS buffer (control) for 30 min at 4°C. To prevent nonspecific binding, 10% goat serum was added after washing twice. Detection of IgM or IgG binding was performed by further incubating with FITC-conjugated goat anti-human IgM (µ chain-specific) and IgG (γ chain-specific) (Invitrogen, Carlsbad, CA) for 30 min at 4°C. Flow cytometric data acquisition was performed with the LSR II (BD Biosciences, San Jose, CA). Binding of IgM and IgG was assessed using relative mean fluorescence intensity (MFI), which was calculated as follows: *Relative*

MFI = (actual MFI) / (MFI of secondary antibody only, in absence of serum)
Post-Tx relative MFI was compared to levels measured in pre-transplant serum.

STATISTICAL ANALYSES

Continuous variables are expressed as mean±SD, and compared using the Student t-test. The difference in survival between Group A and B was calculated using the log-rank test. p-values <0.05 were considered to indicate a statistically significant difference. All analyses were performed with GraphPad Prism 4 for Macintosh (GraphPad Software, La Jolla, CA).

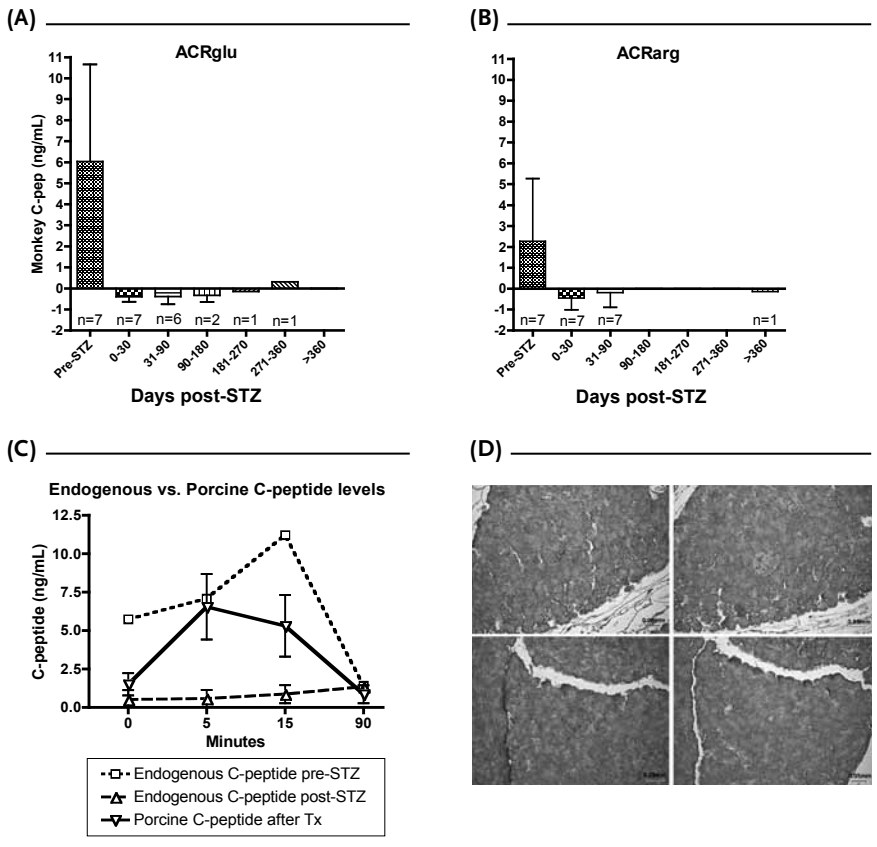

FIGURE 3 | DIABETES INDUCTION BY I.V. STREPTOZOTOCIN: Absence of an endogenous acute C-peptide response (ACR) after induction of diabetes in monkey recipients, when metabolically challenged with (A) intravenous glucose (glu) or (B) arginine (arg). (C) Endogenous and porcine C-peptide levels during IVGTT in Monkey 7273, the islet recipient followed-up for over one year. Data points are mean ± SD of N=7 tests. The last test was carried out three days prior to euthanasia. (D) Left sets of images: glucagon immunostaining of pancreatic tissue analysed in Monkey 7273, right panels show insulin immunostaining of consecutive sections. (Color figure in appendix)

Results

CONFIRMATION OF DIABETES INDUCTION

Diabetes was successfully induced in all monkeys. After STZ, for a mean diabetic period of 24d, continuous i.v. insulin infusion of 1.20±0.41 IU/kg/24h was required to maintain an average blood glucose level of 186±31 mg/dL and to prevent ketoacidosis. Fasting monkey C-peptide levels were reduced from 4.82±1.66 ng/mL to 1.01±0.20 ng/mL (n=9). Moreover, ACR_{Glu} and ACR_{Arg} became undetectable after STZ in all monkeys, and remained undetectable during follow-up (Figure 3A-C). These data indicate that a background level of monkey C-peptide was detectable only with ultra-sensitve RIA kits, and that this level failed to increase after physiologic and non-physiologic stimuli, making it highly unlikely that any native monkey beta cell function was contributing to glucose control, as confirmed by virtually absent insulin staining of post-mortem monkey pancreata (Figure 3D).

OUTCOME OF ISLET TRANSPLANTATION

Functional porcine islet survival, determined by detectable porcine C-peptide in combination with a >50% reduction of exogenous insulin needs, was achieved in all monkeys. In Group A monkeys transplanted with non-Tg islets, islet survival lasted for 7, 20, 31, and 46 days. Insulin-independent normoglycemia was achieved in three of four monkeys for periods of 5, 17, and 36 days, respectively (Figure 4A). In Group B monkeys, the use of hCD46 islets significantly prolonged functional porcine islet survival to the full 3 months of the planned experiment (p=0.0042), or beyond 1 year of follow-up, respectively. This exception was randomly introduced to test the durability of the positive effects of treatment. Four of five Group B monkeys became insulin-independent after Tx for 87, 91 92, and 396 days, respectively (Figure 4B). One of these four recipients gradually lost normoglycemia, which was regained after a re-Tx. In the fifth Group B monkey, insulin-independence could not be achieved with two islet infusions. Nevertheless, even in this animal, exogenous insulin needs were reduced >50% for 3 months with detectable porcine C-peptide.

During times of insulin-independence, fasting blood glucose values were well controlled (Group A: 91±18 mg/dL; Group B: 112±22 mg/dL, p=0.250). Post-Tx weekly fasting porcine C-peptide levels were comparable for Groups A and B

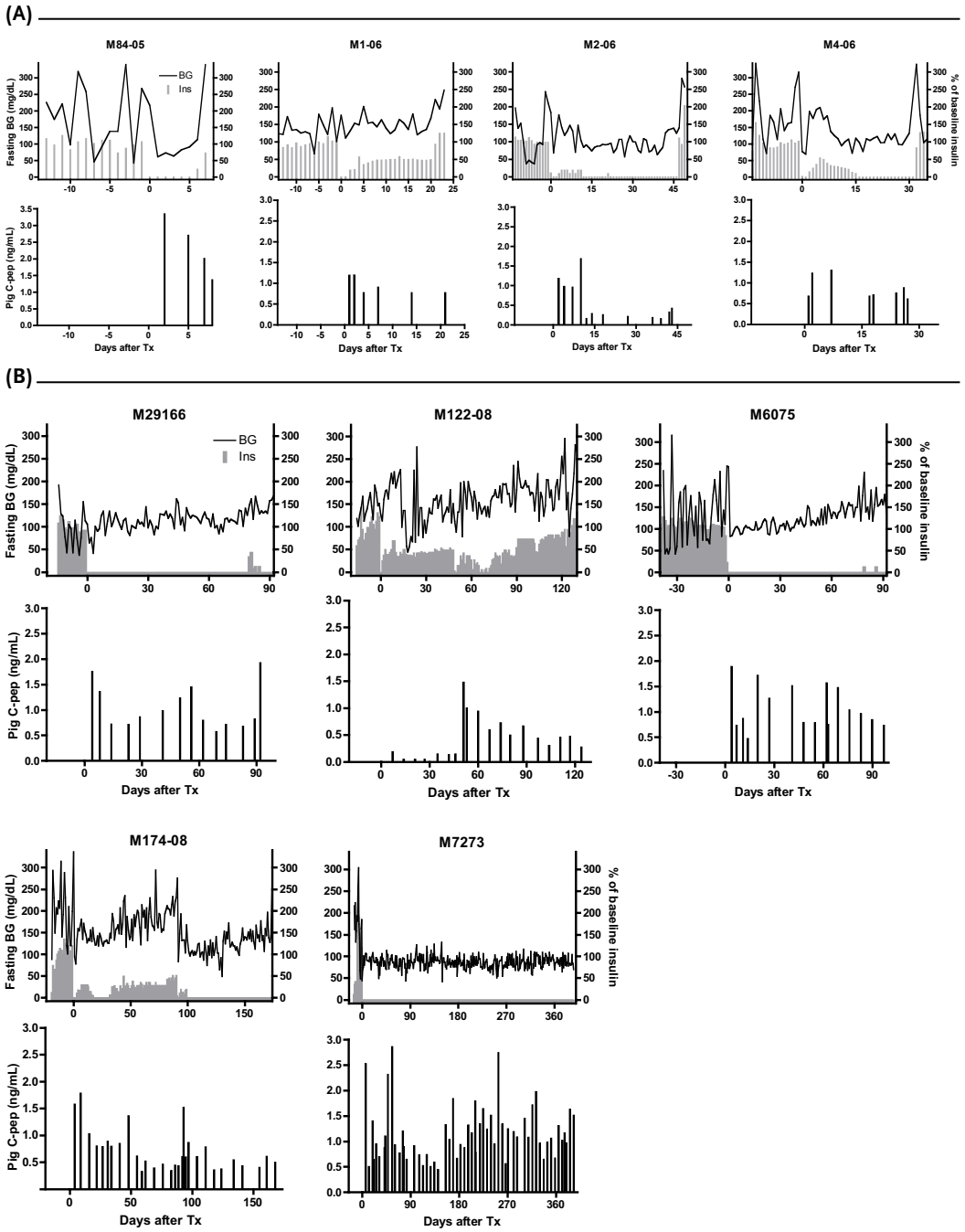

FIGURE 4 | RESULTS OF ISLET XENOTRANSPLANTATION: Fasting blood glucose, exogenous insulin administration as percentage of pre-transplant insulin needs, and porcine C-peptide levels in Group A (A) and Group B (B) monkeys. BG = fasting blood glucose; Ins = exogenous insulin

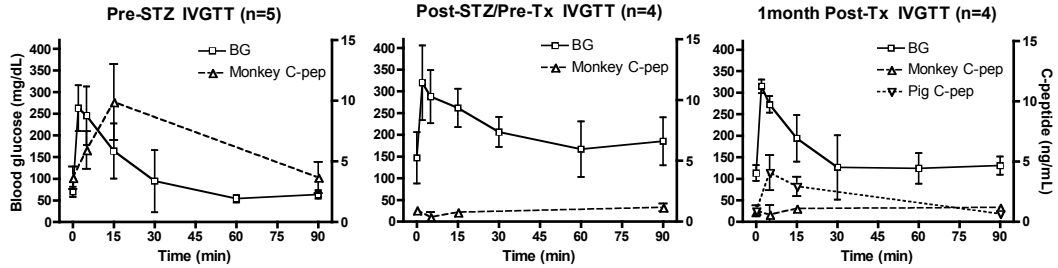

FIGURE 5 | INTRAVENOUS GLUCOSE TOLERANCE TESTS (IVGTT): IVGTT in Group B monkeys before STZ (Pre-STZ), after STZ (Post-STZ/Pre-Tx), and 1 month after transplantation if normoglycemic. Additional data obtained during IVGTT and arginine stimulation tests (AST) are presented in Table 3.

Table 3 Serum acute C-peptide responses (ACR) of pig C-peptide in ng/mL, after metabolic challenges with intravenous glucose (glu) and arginine (arg).

MONTHS		1	2	3	6	7	10	>12
Group A								
M84-05	Day ACRglu ACRarg	ND						
M1-06	Day ACRglu ACRarg	21 0.02						
M2-06	Day ACRglu ACRarg	27 0.50	43 0.18					
M4-06	Day ACRglu ACRarg	24 0.39	27 0.35					
Group B								
M29166	Day ACRglu ACRarg		41 1.67	50 0.46	89 0.77			
M122-08	Day ACRglu ACRarg	ND						
M6075	Day ACRglu ACRarg		33 2.33	63 1.88				
M174-08	Day ACRglu ACRarg	27 2.28	31 0.99			182 1.42*		
M7273	Day ACRglu ACRarg	26 4.22	32 3.39	56 3.70	61 2.68	169 2.87	210 3.45	271 5.77	372 5.58	376 2.82

*ND = Not determined; *Determined after re-Tx*

Table 4 Development of adverse events, body weight.

Recipient ID	Adverse event related to immunosuppression	Adverse event unrelated to immunosuppression	CMV PCR	Final weight as % of Pre-Tx
Group A				
M84-05	-	Euthanasia at day 7 for acute gastric dilatation	positive	100%
M1-06	-	-	negative	105%
M2-06	Diarrhea requiring reduction of MMF dosage	-	positive	88%
M4-06	-	-	positive	90%
Group B				
M29166	Diarrhea with occult blood due to MMF intolerance; MMF discontinued on day 4. At necropsy, microscopic thrombotic lesions in brain and lungs.	Mild progressive kidney failure due to STZ.	negative	107%
M122-08	-	-	negative	107%
M6075	-	-	negative	116%
M174-08	-	-	negative	119%
M7273	-	-	negative	163%

Pre-Tx = Pre-transplantation

during the first 45 days (1.10±0.41 ng/mL versus 1.19±0.88 ng/mL, p=0.860). After 45 days, C-peptide positivity was maintained at 0.87±0.41 ng/mL, but only in Group B recipients. None of the monkeys recovered endogenous beta cell function, evidenced by the absence of primate C-peptide response to arginine and glucose challenge post-STZ (Figure 3).

Post-Tx responses to stimulation with i.v. glucose and arginine showed an evident porcine C-peptide response of engrafted pig islets in Group B, in the absence of a response by native monkey beta cells (Figure 5 and Table 3).

ADVERSE EVENTS

Monkeys remained healthy during the course of each experiment; except one Group A monkey that was euthanized 7 days after Tx with acute gastric dilatation, a not uncommon condition in laboratory animals (23). Adverse events were minimal and the majority of monkeys maintained or gained weight (Table 4). In three Group A monkeys, although free from clinical signs except mild weight loss, reactivated CMV was detectable in serum by PCR. Aspirin may have effectively prevented thrombo-embolic complications potentially associated with anti-CD154 treatment, except possibly in one case (Table 4).

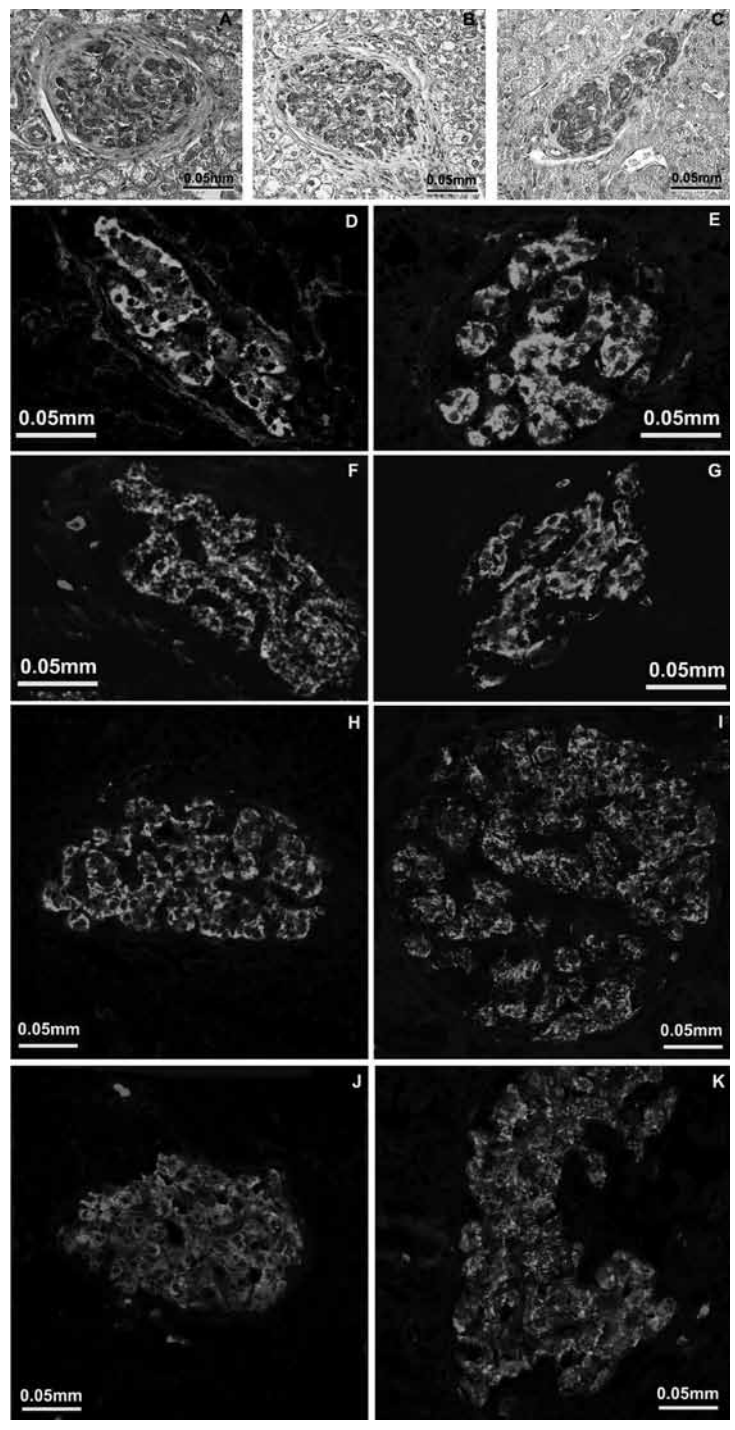

FIGURE 6 | POST-TRANSPLANT HISTOLOGY: (A-C) Insulin immunostaining of liver sections in three monkey recipients after pig hCD46 islet transplantation. (A and B) M29166 and M6075 at three months after transplantation; (C) M7273 >1 year after transplantation. (D-K) Immunofluorescence staining of the liver of monkey recipients following islet transplantation. In all panels insulin is stained green. IgG staining (red) of an islet from a monkey recipient of WT pig islets (Group A) (D) and hCD46 pig islets (Group B) (E). C4d staining (red) of an islet from a monkey recipient of WT pig islets (Group A) (F) and hCD46 pig islets (Group B) (G). Macrophage immunostaining and CD97 positive cells in Group A (respectively H,J) and in Group B (I,K). (Color figure in appendix)

POST-TRANSPLANTATION HISTOLOGY

In Group B monkeys, histological evaluation of post-Tx livers revealed many viable porcine islets (Figure 6A-C) which maintained expression of the Tg even >1 year after Tx (Figure 1D). T cell infiltration was not observed, confirming previous studies in which costimulation blockade was applied (9, 10).

Macrophages and CD97 positive cells were sporadic in both groups (Figure 6H-K). IgG and C4d immunostaining for local antibody binding and complement activation was detected on and around the islet cells in the liver of Group A monkeys; in Group B livers, although IgG was observed, C4d was virtually absent (Figure 6D-G).

ABSENCE OF INCREASE IN XENOREACTIVE ANTIBODY TITERS

Natural anti-pig (anti-Gal + anti-nonGal) and/or anti-nonGal antibodies were present in monkey sera (mainly of IgM isotype), but the extent of binding to pig peripheral blood mononuclear cells (PBMC) did not increase in either Group A or B monkeys after Tx or even after re-Tx (Figure 7).

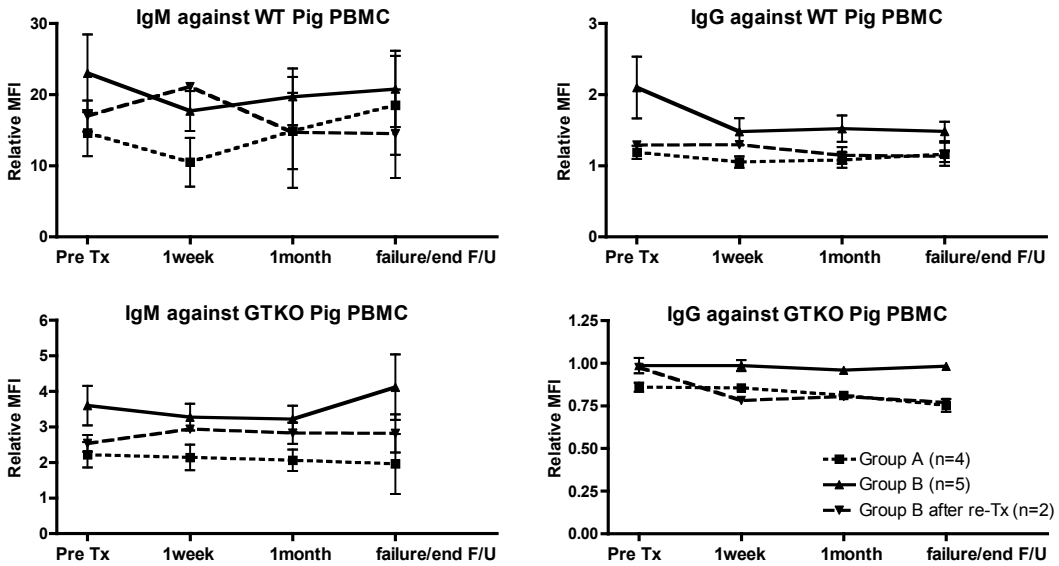

FIGURE 7 | SERUM LEVELS OF XENOREACTIVE ANTIBODIES IN GROUP A AND B MONKEYS: Antibody levels against WT and α1,3-galactosyltransferase gene-knockout (GT-KO) peripheral blood mononuclear cells were determined by flowcytometry before, at 1 week, and at 1 month after islet transplantation, and finally at the time of graft failure or end of follow-up (F/U). No serum antibody sensitization was observed in any monkey recipients. Re-transplantation with hCD46 islets in 2 monkeys did not lead to a serum antibody response.

Discussion

This is the first time in which the survival of a functional islet xenograft was documented for more than one year (at which time the experiment was electively terminated) in a chemically-diabetic monkey, which was neither limited in diet nor received exogenous insulin injections. Transgenic expression of hCD46 on adult porcine islets, when transplanted intraportally into diabetic, immunosuppressed NHP, demonstrated an advantage over not-genetically-modified WT pig islets.

Encouraging results reported by others were achieved using donor islets from WT pigs, suggesting that natural antibodies against Gal may not be too harmful to islets (9,10), yet their potential role in the context of islet xenoTx has recently been reconsidered (24). Although the levels of antibody binding against pig cells did not increase during our experiments, natural antibodies against Gal and nonGal epitopes were found present. The binding of antibodies was observed on both WT and hCD46 islet grafts, whereas activation of complement was only observed on WT islets, suggesting that hCD46 may have exerted a protective role against humoral rejection, possibly reducing the need for immunosuppression. In fact, our immunosuppressive regimen allowed monkeys to remain healthy, in contrast to reports in which adverse events prematurely terminated many experiments (9, 10). The avoidance of calcineurin inhibitors may have added to the long-term preservation of a functional islet mass (25).

In our study, we used market-age hCD46 pigs (not retired breeders) and did not perform any pre-selection of donor islet preparations, in order to model future clinical applications. Transgenic hCD46 together with an anti-inflammatory and anticoagulant protocol, involving LMW-DS in the perioperative transplantation period, should have contributed to reduce the damage induced by IBMIR. Nonetheless, in both experimental groups we observed acute early islet loss characterized by C-peptide release and hypoglycemia: C-peptide peaks 1 and 2h following islet infusion were similar in both groups (data not shown). All together these maneuvers resulted to be insufficient to significantly prevent IBMIR and consequent cell death. Thus, although high numbers of porcine islets turned out to be necessary, we had to conclude that only a marginal mass of surviving cells is sufficient to sustain normoglycemia. Based on these and on our previous observations on the metabolic compatibility between species, we

anticipate that the islet graft mass sufficient to promote normoglycemia would be substantially lower in pig-to-human versus pig-to-NHP transplants (15, 26).

CD46 is well characterized as a protein with regulatory properties able to protect the host cell against complement-mediated attacks activated via both classical and alternative pathways (27). Its features were maintained when transgenically expressed on pig cells (14, 28). Functional complement inhibition activity was demonstrated *in vitro*, whereby both PBMC and aortic endothelial cells showed significantly greater protection from complement-mediated cytotoxicity than cells from WT or GT-KO pigs (28). Fetal pig islets procured from early lineage progenitors of this same hCD46 Tg line, were protected from antibody-mediated destruction in a pig-to-mouse xenograft model (29). Although hCD46 kidneys were resistant to hyperacute rejection when transplanted into baboons (14), Tx of vascularized solid organs from these pig lines could not overcome other xenoTx barriers, such as the occurrence of thrombotic microangiopathy. However, our study seems to indicate that hCD46 expression benefits cell Tx more substantially than organ Tx, especially when cells are transplanted directly into the blood stream.

The small number of animals we were able to study so far is an objective limitation to further speculate on the protective mechanisms of hCD46, but our pre-clinical study certainly offers the best available contest for testing protocols with potential importance for clinical applications.

In conclusion, our study is the first to show how genetic alterations of donor pigs, tailored to combat complement-mediated xeno-islet injury, contribute to long-term function of islet xenografts. With the development of modified anti-CD154 antibodies that may prevent the risk of thromboembolism (30, 31), or newly generated antibodies with equal efficacy, a safer immunosuppressive regimen might become available for use in human Tx, significantly advancing the field of islet xenoTx toward clinical application. Any hypothetical risk from porcine endogenous retrovirus (PERV) has been largely discounted, as more than a decade of focused research has not demonstrated any *in vivo* transmission of PERV to NHP or human recipients, even after prolonged exposure to porcine tissues (32, 33).

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ANATOMICAL SITES

CHAPTER 5

The choice of anatomical site for islet transplantation

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ABSTRACT

Islet Tx into the portal vein is the current clinical practice. However, it has now been recognized that this implantation site has several characteristics that can hamper islet engraftment and survival, such as low oxygen tension, an active innate immune system, and the provocation of an inflammatory response (IBMIR). These factors result in the loss of many transplanted islets, mainly during the first hours or days after Tx, which could in part explain the necessity for the Tx of islets from multiple pancreas donors to cure T1D. This increases the burden on the limited pool of donor organs. Therefore, an alternative anatomical site for islet Tx that offers maximum engraftment, efficacious use of produced insulin, and maximum patient safety is urgently needed. In this review, the experience with alternative sites for islet implantation in clinical and experimental models is discussed. Subcutaneous Tx guarantees maximum patient safety and has become clinically applicable. Future improvements could be achieved with innovative designs for devices to induce neovascularization and protect the islets from cellular rejection. However, other sites, such as the omentum, offer drainage of produced insulin into the portal vein for direct utilization in the liver. The use of pigs would not only overcome the shortage of transplantable islets, but genetic modification could result in the expression of human genes, such as complement-regulatory or 'anticoagulation' genes in the islets to overcome some site-specific disadvantages. Eventually, the liver will most likely be replaced by a site that allows long-term survival of islets from a single donor to reverse T1D.

Introduction

The islets of Langerhans are scattered throughout the exocrine tissue of the pancreas, providing the organ with its specific anatomical architecture. The development of a reproducible automatic method for islet isolation (1) created the possibility for islet Tx to reconstruct natural glucose homeostasis in patients with T1D, while avoiding the Tx of the exocrine tissue. Today, clinical islet Tx into the portal vein can be successful in experienced centers (2-4).

However, after the initial promising results of the Edmonton protocol (5), long-term graft function has been rather disappointing, and the successes proved hard to replicate in some other centers (4, 6). In addition to auto-immune and allo-immune barriers, the survival of an islet transplant depends on the diffusion of oxygen and nutrients provided by the particular site of Tx, and on the revascularization of the graft at that site. Ideally, the optimal site would allow for maximum islet survival, maximal efficacious use of the produced insulin, and maximum patient safety. It has now been recognized that the liver may not provide the conditions that can meet these requirements. The future of islet Tx will in part depend on identifying a Tx site that can allow for long-term engraftment of islets from a single donor to reverse T1D.

Since the first islet Tx experiments, many other sites than the liver have been explored, with various success rates. In humans, islets have been transplanted under the kidney capsule, into the spleen, into the peritoneal cavity, into the muscles of the abdomen and forearm, and under the skin (7-11). In rodent and in large animal models, various sites have been explored in order to overcome problems that can hamper islet engraftment; these include mechanical stress, immune activity, inflammatory responses, oxygen and nutrient supply, and clinical feasibility. Although many experiments were carried out, as we now know, under suboptimal technical conditions or suboptimal or harmful immunosuppression, it is still worthy to review these experiences in our search for the optimal site for islet Tx.

This review will briefly discuss the advantages and disadvantages of the liver and other sites for islet Tx in human and animal research, with relevance for future clinical application.

The Liver as the Site for Islet Transplantation

HISTORICAL PERSPECTIVE

The first described attempt to transplant pancreatic tissue for the treatment of diabetes was performed in 1893 (12), almost 30 years before the discovery of insulin. In the following 75 years, until a pancreas digestion method using collagenase was developed, many animal experiments related to Tx of whole pancreatic tissue rather than isolated islets. The sites of Tx included spleen, liver, peritoneal cavity, omentum, retroperitoneum, subcutaneous tissues and gastric submucosa (13, 14). It was also recognized that tissue Tx offers the possibility for Tx into immunoprivileged sites, such as the anterior chamber of the eye and the testis (15-17).

These reports indicated that the search for the ideal site for islet Tx is not new. However, many early studies reported poor outcomes in terms of reversal of diabetes or recipient survival because of the co-transplanted exocrine tissue that caused complications, such as autodigestion and fistula formation. To address this problem, rodent studies of Tx of fetal or neonatal pancreatic tissue were performed (17), since the exocrine component is still underdeveloped at these ages and the endocrine component still has the potential to grow. Early attempts with intramuscular Tx of human fetal pancreatic fragments were relatively successful, with improvement in glucose control in three of 13 patients without immunosuppression (18).

With the development of the method of pancreas digestion using collagenase in 1967, rat islets could be isolated from the exocrine tissue (19) and, if transplanted into the peritoneal cavity of allogeneic diabetic rats, reduce blood glucose levels and polyuria (20). In subsequent experiments, normoglycemia could be restored if the islets were transplanted into the portal vein of syngeneic rat recipients (21). The results of intraportal Tx were significantly superior to intraperitoneal and subcutaneous Tx (22). Also, in human patients, the first - albeit modest - successes of islet autoTx after pancreatectomy for chronic pancreatitis were obtained after intraportal infusion (23).

When the automatic method for islet isolation was introduced in 1986 (1), the collection of human islets in consistent yields sufficient for clinical Tx became

a reality. This led to the first clinical successes of intraportal islet alloTx (24). Subsequent improvements in outcome depended mainly on the introduction of a steroid-free immunosuppressive protocol and the Tx of islets from more than one donor (5).

THE RATIONALE FOR INTRAPORTAL ISLET TRANSPLANTATION

In normal non-diabetic conditions, insulin from the pancreatic beta cells is secreted directly into the portal venous system and is metabolized largely in the liver (25). It was therefore reasoned that the liver may provide a physiologic environment for islets that enables the effective use of the secreted insulin (21). The promising results of the first experimental and clinical transplants appeared to confirm this hypothesis (22). It was recently reported that the insulin produced by islet allografts in type 1 diabetic patients was utilized in the liver in a similar way to insulin produced by non-diabetic control subjects, with 80% being metabolized in the liver (26).

Furthermore, intraportal infusion can today be performed by a minimal invasive procedure under local anesthesia after sedation, in an outpatient setting. Access to the portal vein is obtained by a percutaneous transhepatic approach under ultrasound or fluoroscopic guidance (27, 28). During the infusion, the portal venous pressure is monitored. Although not entirely free from complications, such as portal hypertension and bleeding, this procedure can be considered safe and efficacious (28, 29). Moreover, its minimal invasive characteristics allow for repetitive infusions in the event that a first transplant does not result in normoglycemia.

UNFAVORABLE CONSEQUENCES OF INTRAPORTAL ISLET TRANSPLANTATION

Although the results of clinical intraportal islet Tx have been superior over other sites, several characteristics of the liver and the portal venous system can hamper islet engraftment and survival. After intraportal Tx, the islets that were richly vascularized by arterial blood in the pancreas, now depend on oxygen diffusion from the hypo-oxygenated portal venous blood (pO_2 10-15mm Hg vs. 40mm Hg in arterial blood [30]), until revascularization by the hepatic arterial system (31, 32). It has been well demonstrated that hypoxia is an important apoptosis-inducing signal in beta cells (33).

The portal system is the drainage system to the liver for many toxins that are absorbed in the intestine, such as lipopolysaccharides (LPS) (34). This is most likely an important reason why the liver has an active innate immune system. In particular, liver macrophages (Kupffer cells) play a key role in the early

inflammatory events that occur after islet Tx (35), and may induce a subsequent adaptive immune response (36). Tx into this immuno-active site is in contrast with the desire for immune hyporesponsiveness after Tx.

Portal blood is hyperglycemic compared to systemic blood, and chronic hyperglycemia can have toxic effects on the islets (37), although it should be noted here that toxic levels may not be reached in portal blood. In addition, the levels of orally-administered immunosuppressive agents are higher in the portal blood than after having passed through the liver (first pass effect), and it is debatable whether these high levels protect the grafted islets or have harmful effects. Almost all currently-used immunosuppressive drugs have an adverse effect on beta cell function and survival (34, 38).

Furthermore, the IBMIR is a major islet-disrupting event that takes place when islets are infused into the bloodstream (39). The IBMIR is triggered by the exposure of islet surface molecules, such as TF, and the production of inflammatory cytokines, possibly as a result of mechanical and chemical stress during the isolation process (40). Upon direct contact with blood, this results in activation of coagulation, platelets, and complement, and the attraction of granulocytes, leading to islet disruption (39). It is likely that it occurs not only after intraportal Tx, but after any intravascular infusion, such as into the splenic vein.

It has been investigated whether transplanted islets produce glucagon in response to hypoglycemia and thus improve hypoglycemia-unawareness, a major indication for islet Tx. It is thought that this response is impaired due to the lack of innervation of the grafted islets. This impairment was larger after intraportal Tx compared to intraperitoneal Tx in pancreatectomized dogs (41). Because the liver is the site of endogenous glucose production, higher intrahepatic glucose levels could possibly prevent the islets from sensing peripheral hypoglycemia (42).

Last, histological follow-up of graft survival or rejection is impossible after intraportal islet infusion, as the islets will be randomly distributed throughout the portal vascular system. However, this problem may soon be overcome by the development of non-invasive imaging methods, such as magnetic resonance imaging (MRI) and positron-emission tomography (PET), to monitor the islet grafts (43, 44).

Alternative Sites for Islet Transplantation

ISLET TRANSPLANTATION INTO THE RENAL SUBCAPSULAR SPACE

In murine models, the renal subcapsular space is the most common site for Tx of islets to study immune responses, drug effects, and the result of genetic modifications (Table 1 and Figure 1). The diabetes reversal rates are often high, even with limited numbers of islets. Tx into a non-obese diabetic mouse without an effective immune system, the so-called NOD-SCID mouse, has become the gold standard for islet quality control after the islet isolation process for most large animal and clinical studies. However, in large animals and humans, investigations of islet Tx beneath the kidney capsule are limited, most likely due to the initial success of intraportal Tx. Early attempts did not lead to any improvement in the diabetic state, possibly because of a limited blood supply in the early post-Tx phase until revascularization takes place (7, 45, 46). Also, later clinical results, although still before the introduction of the Edmonton protocol, were inferior to Tx into the liver (47).

ISLET TRANSPLANTATION INTO THE SPLEEN

Another option for intravenous Tx is retrograde infusion into the splenic vein. In animal studies, Tx into the spleen offers the possibility of histological investigation after splenectomy, while keeping the recipient alive (48). It was also hypothesized that a greater tissue volume could be infused without the risk for portal hypertension (49), a known complication of intraportal infusion. The spleen is well-perfused with oxygenated blood and drains the released insulin into the portal circulation. In contrast, it can be expected that islet damage due to inflammation (IBMIR) takes place after intrasplenic Tx, comparable with intraportal Tx.

Models of pancreatectomy and autoTx allow for studies of the site of Tx in the absence of immunologic barriers. In dogs, equal or even better results were achieved when the spleen was compared to the liver (46, 49, 50). In clinical alloTx, one case was described of insulin-independence, although it concerned the Tx of pancreatic microfragments from a juvenile donor (8). A new evaluation of the spleen for islet Tx, however, will need to prove advantageous over the liver, for which a much broader experience has been established.

SUBCUTANEOUS ISLET TRANSPLANTATION

From the viewpoint of patient safety, islet Tx under the skin is an attractive option. The site is easily accessible for placement of the islet mass, allows for biopsies to monitor islet viability, and makes removal of the graft, if necessary, and re-Tx possible, all with the use of only minimally-invasive procedures. A disadvantage is that the released insulin can reach the liver only via the systemic circulation. Although exogenous insulin therapy is able to control blood glucose values, islet Tx with portal delivery of insulin is believed to be optimal for glucose metabolism. Moreover, subcutaneous islets became necrotic after alloTx in humans (10) and in mice (51), indicating suboptimal vascularization and insufficient oxygenation and nutrient supply during the first few days after Tx. Compared to the subcutaneous tissue, the muscle may provide better conditions for revascularization; successful engraftment of autotransplanted islets into a pediatric recipient has been reported (9).

Alternatively, to address the problem of insufficient oxygenation, studies in mice and rats have aimed at the prevascularization of the subcutaneous area by the implantation of biomaterials. Cylinders, bags, or mesh devices were placed under the skin 7 to 40 days prior to Tx to induce the creation of a vascular bed (52-55). In some cases, the biomaterials were designed to release growth factors, such as fibroblast growth factor, to stimulate angiogenesis (51, 56, 57). After this period, the device was replaced with the islet graft or the islets were placed inside the device. In all studies, the rate of reversal of diabetes was superior to that in control subjects without prevascularization.

In addition, the placement of islets inside a device could mechanically immuno-isolate the islets, reducing or preventing cellular rejection, the dominant mechanism of islet rejection (58). In a controversial report, xenoTx of neonatal pig islets in adolescent patients with T1D was described, resulting in reduced insulin requirements in half of the 12 subjects and temporary insulin-independence in two, without the use of immunosuppression at any time (59). It is not clear whether the reported beneficial effects were related to the chamber or to the co-transplanted Sertoli cells, that can have an immunomodulating effect (60, 61).

With these new developments, subcutaneous islet Tx may prove to be a promising technique. In particular, in the case of clinical xenoTx, it could offer a controllable setting for this therapy; however, this work is still very much experimental.

Table 1 Advantages and disadvantages of investigated sites for islet transplantation.

	Site	Highest species tested	Main advantage(s)	Main disadvantage(s)
1.	Liver	Human	- Proven clinical efficacy - Safe, minimally-invasive - Physiological use of insulin in liver	- Early loss because of IBMIR and hypoxic apoptosis - Active (innate) immune system
2.	Renal capsule	Human	- Well-established rodent model	- Tight capsule in large animals - Poor initial clinical results - Systemic release of insulin
3.	Spleen	Human	- Reduced risk of portal hypertension - Physiological use of insulin in liver	- Lack of clinical experience - IBMIR
4.	Skin	Human	- Easy implantation - Easy to biopsy - Easy to re-transplant - Maximum patient safety	- Poor vascularization unless pre-vascularization is initiated - Systemic release of insulin
5.	Omentum	Non-human primate	- Physiological use of insulin in liver - Rich vascularization and tissue remodeling capacities	- Risk of adhesions, internal hernia, and ileus
6.	Intestinal/gastric submucosa	Pig	- Rich vascularization - Physiological use of insulin in liver - Endoscopic approach possible	- Large animal data still limited
7.	Pancreas	Mouse	- Physiologic micro-environment - Possibly improved exocrine function	- Risk of induction of pancreatitis
8.	Brain/Testis/ Eye/Thymus	Dog/Mouse Mouse/Pig	- Immunoprivilege - Re-education of immune system	- Limited clinical applicability
9.	Muscle	Human	- Rich vascularization	- Systemic release of insulin

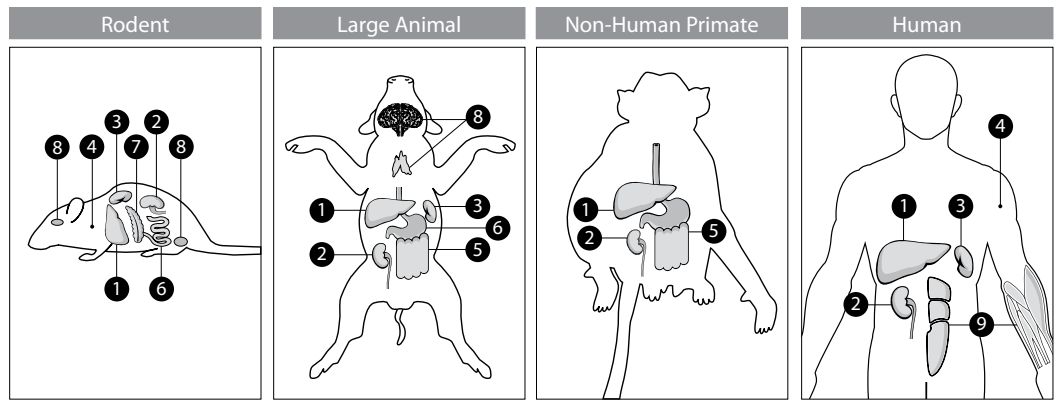

FIGURE 1: Experience with various sites for islet transplantation in small and large animal models and in humans. Numbers in the figure correspond to numbers in Table 1 in which the advantages and disadvantages of each site are elaborated.

ISLET TRANSPLANTATION INTO OMENTAL POUCHES

Renewed interest in transplanting islets into a pouch created in the omentum has been shown in syngeneic rat studies (62). When applied in dogs after total pancreatectomy and autoTx, 6 out of 6 animals obtained good islet function (63), whereas earlier experiments reported only 50% success (49, 64). Islets engrafted in the omentum respond in a more physiological pattern than when transplanted under the renal capsule, with respect to glucose metabolism and insulin sensitivity (65). This effect is due to the direct drainage of insulin into the portal circulation. Other favorable circumstances in relation to the omentum are good vascularization and the ability to induce tissue repair, as has been applied in the treatment of myocardial ischemia and in reconstructive plastic surgery through omental transposition (66).

Very recently, the results of alloTx in a NHP model were presented, and were encouraging; islets were transplanted into the omentum, which was then sutured between the muscular layers of the abdominal wall (67). However, caution needs to be applied to this technique in a clinical setting, as the omentum can be a source of adhesions after surgical manipulation that can lead to intestinal strangulation, ileus, and internal hernia (68). Because of the likely occurrence of adhesions, we believe that islet Tx into the peritoneal cavity, as was attempted in early days (11), may not be a site of future interest.

ISLET TRANSPLANTATION INTO THE GASTRO-INTESTINAL TRACT

The submucosal space of the gastrointestinal tract has several characteristics that could favor islet Tx into this site - a similar embryonic origin as the pancreas, rich vascular supply of oxygen and nutrients, and venous drainage into the portal blood stream. In culture, the intestinal submucosal matrix can provide a trophic environment for isolated islets (69). A study of Tx in syngeneic hamsters demonstrated that islets can survive in this space and reverse diabetes (70). A preliminary report of a large animal model demonstrated that autotransplanted islets can engraft in the gastric submucosal space of minipigs (71). The islets were able to execute an insulin response following an arginine challenge four weeks after Tx (71).

The most physiological and therefore perhaps most supportive microenvironment for islets is the pancreas itself. Mouse islets retrieved one month after syngeneic Tx directly into the pancreas retained the ability to release insulin in response to a glucose stimulus, while this response was absent in islets that had been transplanted into the liver (72). In return, the hormone release by islets may ameliorate the exocrine function of the pancreas

that is often impaired in diabetic patients (73). Prior to translation to a clinical application, it will be necessary to investigate the efficacy and safety of this procedure regarding cure of diabetes without induction of pancreatitis in larger animal models.

IMMUNOLOGICALLY PRIVILEGED SITES AND TOLERANCE

Tx of cells rather than whole organs introduces options for placement into sites where there is a reduced, absent, or suppressed immunological response, so-called immunoprivileged sites, such as the testis, brain, and anterior chamber of the eye. It was originally thought that immune privilege results from ‘immunologic ignorance’ (74), but subsequent studies have shown that the mechanisms of immune privilege are much more complex and can involve Fas - Fas ligand interactions, an immune response deviated toward a Th2-like response (75, 76), and failure of infiltrating T cells to acquire a direct cytotoxic function (77). In a carefully conducted rodent study, it was demonstrated that islet Tx into the testis induced $CD4^{+}CD25^{+}$ regulatory T cells that were essential to maintain a tolerant immune state (78). Although these studies can provide great insight into the mechanisms of immune privilege, their clinical relevance may be rather low. This will hold as well for Tx into the anterior chamber of the eye (79) or into the brain (80).

Islet Tx into the thymus was attempted with the goal to re-educating the immune system, leading to tolerance (81). Islets survived in that site, but the recipients still required immunosuppressive therapy and the mechanisms of tolerance were not studied extensively (81). More information may soon become available from studies using vascularized thymic tissue in combination with islet and kidney Tx (82).

Can Xenotransplantation at Different Donor Ages Overcome Site-specific Problems?

The pig offers greater opportunities than humans to select a donor at an age when the islets have favorable properties; islet-like cell clusters isolated from fetal or neonatal piglets retain the ability to mature and proliferate (83), are believed to be less immunogenic than adult islets, and are more resistant to hypoxia (84). As a result of these characteristics, these types of islets may have a better survival potential at different recipient sites. In the liver of NHPs survival and function of neonatal pig islets has been demonstrated (85). In a pilot study more than a decade ago, fetal pig islets were at least demonstrated to survive in humans and secrete some insulin and C-peptide (86).

The Tx of porcine embryonic pancreatic tissue of early gestational age (29-56 days of 120 days total gestation) can avoid islet isolation procedures that can have harmful effects on the islets in the form of hypoxic, mechanical and chemical stress. In embryonic tissue, the exocrine compartment remains undeveloped, and will develop only to a minimal extent after Tx into the rat omentum (87). In contrast, the endocrine component has been shown to survive, expand, mature, and produce porcine insulin when transplanted under the kidney capsule of nude mice (88) or into the omentum of diabetic rats (87). The latter has the advantage that insulin is delivered to the liver (87). This type of pig tissue can mature after Tx into omental pouches or after implantation into the mesentery of cynomolgus monkeys (89, 90). More data on the ability of embryonic tissue to reverse diabetes in NHPs are eagerly awaited. A major disadvantage of this approach is that it takes weeks to months before the tissue matures and expands to produce insulin in sufficient quantities to control glucose metabolism. During this period, both exogenous insulin therapy and immunosuppression need to be administered to the recipient.

The pig may offer a solution to the increasing need for transplantable islets. Pigs can provide an unlimited source of islets in quantities that may satisfy the metabolic requirements of diabetic patients, and that are able to produce insulin that functions in humans (91). The reported six-month

survival of adult pig islets transplanted intraportally into NHPs is promising (92), although clinical application will require significant modification to the immunosuppressive regimen.

Finally, the pig offers opportunities for genetic modification that may help overcome some of the unfavorable site-specific conditions. For example, IBMIR may be reduced or prevented by islets from pigs Tg for a human CRP, hCD46 (membrane cofactor protein), or for human tissue factor pathway inhibitor, human thrombomodulin, or human CD39, for 'anticoagulation' (93). Islets from neonatal GT-KO piglets are free of Gal expression, whereas WT neonatal islets express Gal, which may be a target for xenoreactive natural antibodies (94).

Conclusions

Although Tx into the liver is the current practice for islet alloTx, it is now recognized that the liver may not provide the conditions favoring optimal islet survival. In particular, the major early loss of islet mass from IBMIR requires the Tx of islets from more than one donor to achieve normoglycemia. In addition, the islets are exposed to an active immune system, higher glucose and immunosuppressive drug levels, and hypoxic conditions in the portal circulation. Many alternative sites have been explored; some in experimental settings aimed at clarifying immunological or biological processes, others aimed at providing an alternative for clinical Tx. Eventually, the liver will most likely be replaced by a site that can allow long-term survival of islets from a single donor to reverse T1D. The pig can provide additional options as an islet donor, perhaps by producing a genetically-modified islet supply adjusted for the conditions at a particular transplant site.

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ENDOSCOPIC

CHAPTER 6

Endoscopic gastric submucosal transplantation of islets (ENDO-STI): technique and initial results in diabetic pigs

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ABSTRACT

The results of Tx of human donor islets into the portal vein in patients with diabetes are encouraging. However, there are complications, e.g., hemorrhage, thrombosis, and an immediate loss of islets through the 'instant blood-mediated inflammatory reaction' (IBMIR). The gastric submucosal space (GSMS) offers potential advantages. Islets were isolated from adult pigs. Recipient pigs were made diabetic by STZ. Donor islets were injected into the GSMS through a laparotomy (Group 1A, n=4) or endoscopically (Group 1B, n=8), or into the portal vein through a laparotomy (Group 2, n=3). The pigs were followed for a maximum of 28 days. Monitoring of C-peptide in Group 1 indicated that there was minimal immediate loss of islets, whereas in Group 2 there was considerable loss from IBMIR. In Group 1, there were significant reductions in mean blood glucose and mean exogenous insulin requirement between pre-Tx and 20 days post-Tx. In Group 2, there was no significant reduction in either parameter. Insulin-positive cells were seen in the GSMS in Group 1, but not in the liver in Group 2. Endoscopic gastric submucosal Tx of islets (ENDO-STI) offers a minimally-invasive and quick approach to islet Tx, avoids IBMIR, and warrants further exploration.

Introduction

To determine the optimum site for islet Tx, many anatomic locations have been tested (1). The liver is the most commonly used site, but survival of islets transplanted into the portal vein is suboptimal (Table 1). Approximately one-third of islet recipients experience at least one adverse event within the first year, with almost half being related to the transplant procedure (2, 3); although most resolve without sequelae, almost half require hospitalization (2-7). Occasionally, a mini-laparotomy is required for direct injection into the portal system (8). Exposure to blood results in a substantial loss of islets from the IBMIR (9-12), a pathologic nonspecific inflammatory response to tissues that are not normally present in the blood. The oxygen tension in the portal vein is the lowest in the circulation, contributing to apoptosis of the islets, resulting in a further reduction in the number that survive (13, 14).

The GSMS offers potential advantages over the portal vein (Table 1). There would be minimal risk of bleeding or thrombosis and, as the islets would not be injected directly into the blood, IBMIR would be avoided. Importantly, the GSMS is endoscopically accessible, which would allow islet Tx as a minimally-invasive procedure; re-Tx could readily be carried out on multiple occasions.

In 1930, Badile carried out autoTx of pieces of pancreas in different sites in cats; the longest survival (105 days) was when the pancreas was placed in the GSMS (15). In 1978, Champault et al. used pieces of pancreas as autotransplants and allotransplants in the GSMS of the rabbit; the transplants maintained normal glucose levels for 60 days (16). In STZ-induced diabetic hamsters, in 2002 Tchervenivanov et al. transplanted islets into the duodenal submucosal space, and demonstrated that they could restore a normoglycemic state for >20 weeks (17). Since we began our current study, Caiazzo et al. have reported better engraftment of autotransplanted islets in the GSMS when compared with the kidney capsule in non-diabetic miniature swine (18); the islets were transplanted through a laparotomy. We have explored - to our knowledge, for the first time - the feasibility of alloTx of islets into the GSMS by endoscopy (19), and herein report the technique and our encouraging initial results.

Materials & Methods

ANIMALS

Landrace-large white cross-bred retired breeder sows, weighing approximately 150kg (obtained from a registered pig supplier) (n=9), were used as islets donors. Pigs from the same source (13.3±4.3kg) were recipients of islets (n=11). Because of a relatively high incidence of infectious complications, later experiments used specific pathogen-free Yucatan miniature swine (Sinclair Research Center, St. Louis, MO); retired breeder sows (>70kg, n=2) were the source of islets, and haploid-identical offspring (12.25±1.61kg, n=4) were recipients (to minimize the immune response). In all recipients, intravascular catheters were inserted into both right jugular veins for blood withdrawal and drug infusion; all received cefazolin 250mg i.v. twice daily for 3 days peri-operatively.

All animal care procedures were in accordance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985). All protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

DONOR PANCREATECTOMY AND ISLET ISOLATION

Through a midline abdominal incision (20), the pancreas was perfused *in situ* with Hank's Balanced Salt Solution (HBSS), and pancreatectomy was performed without warm ischemia. The islet isolation procedure has been described previously (20). In brief Liberase-PI (Roche, Indianapolis, IN) or Collagenase-P (Roche) from multiple lots was used for the exogenous enzyme blend and was infused intraductally at 15-20mL/min. Digestion temperature never exceeded 34°C; the degree of dissociation was monitored by dithizone staining and microscopic evaluation of pancreatic samples every minute. Digestion was discontinued by diluting the enzyme solution with cold RPMI-1640 (Life Technologies, Carlsbad, CA), supplemented with 10% heat-inactivated fetal calf serum (Life Technologies). Separation of islet-enriched fractions was performed using discontinuous gradients (at densities of 1.108, 1.096, and 1.037) in a COBE 2991 Cell Separator (Gambro BCT, Lakewood, CO). Purity was evaluated after dithizone staining of islet samples, and expressed as percent of islets/whole tissue. An approximate mean of 350,000 IEQ was obtained from each donor pancreas.

Table 1 Comparison of GSMS and portal vein (intraportal, liver) as sites for islet transplantation.

	GSMS	Intraportal
Embryological derivation	Foregut	Foregut
Arterial supply	Rich	Poor
Venous drainage	Portal	Portal
Oxygen tension	High (>60mm Hg)	Low (<15mm Hg)
Loss of islets from IBMIR	Absent?	Massive
Local IS drug levels/toxicity	Low	High
Access for Tx	Endoscopy	Echo-guided percutaneous venous catheter injection + fluoroscopy + portal venous pressure monitoring, or mini-laparotomy
Biopsy follow-up	Possible	Not possible
Potential complications	Perforation	Intraperitoneal hemorrhage, portal vein thrombosis, steatosis, portal hypertension
Site of ectopic pancreatic tissue	Yes	No
Glucose concentration	Systemic	High

GSMS = gastric submucosal space; IS = Immunosuppression; IBMIR= instant blood-mediated inflammatory reaction.

ISLET VIABILITY AND FUNCTIONAL ASSAYS

Islet viability was assessed by the double fluorescent calcein-AM and PI stain (20, 21). To assess *in vitro* functional properties, islet preparations were subjected to a dynamic secretagogue challenge in a perifusion apparatus. One hundred hand-picked islets were loaded into the perifusion apparatus and conditioned for 30min in Krebs-Ringer bicarbonate buffer (KRBB) supplemented with 0.5% BSA (Fraction V, Sigma-Aldrich, St. Louis, MO) containing 2.8mM glucose. Experimental conditions involved exposure of islets to KRBB with 2.8mM glucose, 20mM glucose, and high glucose plus theophylline (5mM), respectively. Eluates were collected every minute. Insulin concentrations in the eluates were measured by ELISA (Porcine Insulin Kit, Alpco, Salem, NH) and data used for the calculation of stimulation index (ratio between AUC of stimulated and basal insulin release) (20).

To test the functional properties of the isolated islets *in vivo*, diabetic recipient NOD-SCID mice were transplanted with 250-1,000 hand-picked islets (of approximately 150μm in diameter) under the kidney capsule, as described previously (21). Islets from 6 Group 1 donors and from 1 Group 2 donors were tested. All recipients of >350 islets normalized glucose levels within the first week.

INDUCTION OF DIABETES IN THE RECIPIENT

Diabetes was induced by STZ (200mg/kg i.v., Zanosar; Sicor Pharmaceuticals, Irvine, CA) two weeks prior to islet Tx (22). Pigs were fasted overnight and were prehydrated with i.v. 0.9% NaCl (10mL/kg/h) for 1h before STZ administration (to reduce potential nephrotoxic side effects). All pigs received STZ that was reconstituted with 0.9% NaCl and was administered by i.v. injection over 1min. After cell damage, glucose was infused i.v. either continuously or intermittently to prevent severe hypoglycemia; the rate and concentration of the glucose administration depended on the blood glucose level. IVGTTs were performed before and after STZ injection to confirm the diabetic state. Blood glucose levels were checked twice daily, and short-acting or intermediate-acting insulin (Humulin R and Humulin N; Eli Lilly) was administered subcutaneously to control hyperglycemia (i.e., to maintain the blood glucose 200-250mg/dL).

TRANSPLANTATION OF ISLETS

Recipients in Group 1 (GSMS) received a mean of 15,887±6,269 IEQ/kg islets, and in Group 2 (portal vein) 13,333±2,886 IEQ/kg. There was no significant difference in the number of islets transplanted between the two groups.

Group 1A: Islet Tx into the GSMS by laparotomy (n=4)
Through a midline incision, the serosa and muscular layers of the stomach were gripped by two fingers to create an actual space in the virtual GSMS. The islet preparation was injected into the GSMS of the antrum in 4-6 different sites using an 18 gauge needle. The abdomen was closed in layers. The procedure was completed in approximately 30min.

Group 1B: Islet Tx into the GSMS by endoscopy (ENDO-STI, n=8)
As a preliminary study, ink was injected (as described for islets below) into the GSMS in 2 pigs (not included in Group 1). Immediate necropsy and examination of the stomach wall confirmed that the GSMS had been successfully targeted (Figure 1).

Using an 18 French endoscope (Fujinon, Wayne, NJ), the islets were injected via a sclerotherapy needle (Interject 23G M0051815, Boston Scientific, Natick, MA) into the GSMS at 4-6 different sites in the gastric antrum (Figure 2). A 3-way connector (MX4341L Medex, Dublin, OH) was attached to the needle to facilitate delivery of the islets (Figure 2A). Immediately before infusion, the islets were resuspended in 6-8 Eppendorf tubes (Eppendorf, Westbury, NY), each tube containing 0.5mL, including islets and CMRL-1066 (Mediatech, Manassas, VA) supplemented with 1% heat-inactivated recipient serum. The catheter and needle were first primed with 1mL CMRL-1066 supplemented with 5% heat-inactivated recipient serum. To ensure all islets reached the GSMS,

the catheter and needle were flushed with 1mL CMRL-1066 supplemented with 1% heat-inactivated recipient serum. The endoscopic procedure was completed in 5-10min without complications. (In one case, however, it is likely that some islets were not injected into the GSMS [see below].)

Group 2: Islet Tx into the portal vein by laparotomy (n=3)
Through a midline incision, the portal vein was identified. After the administration of systemic heparin (90IU/kg i.v.), the islets (suspended in 10mL CMRL-1066 supplemented with 100IU heparin) were infused directly into the portal vein through a 23 gauge catheter under gravity pressure over approximately 10-20min. The catheter was withdrawn, hemostasis was assured by pressure for 5min, and the abdomen was closed.

IMMUNOSUPPRESSIVE REGIMEN

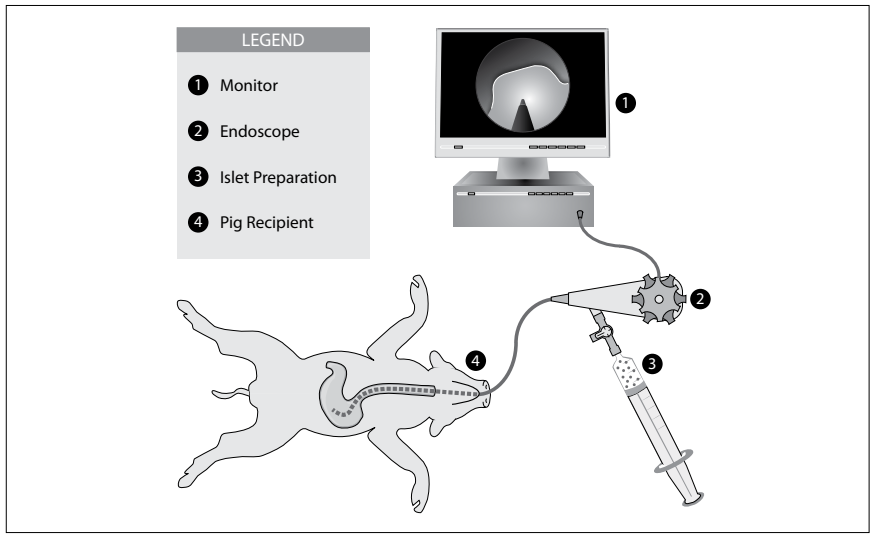
Recipients were administered tacrolimus (0.05mg/kg i.m. twice daily) and MMF (25mg-100mg/kg p.o. twice daily). Blood trough levels were monitored at least twice weekly and maintained at 10-15ng/mL (tacrolimus) and 2-6µg/mL (MMF).

FOLLOW-UP

Two pigs in Group 1B were electively euthanized 24h after islet Tx for histological examination of the islets. In the remaining Group 1 recipients (n=10) (Table 2), monitoring of blood glucose was carried out twice daily with a TrueTrack smart system glucometer (Home Diagnostics, Fort Lauderdale, FL) to measure a fasting/semi-fasting overnight level, and a 4h post-prandial level in

FIGURE 1: Macroscopic appearance of stomach wall after endoscopic injection of ink into the GSMS (as described in the text). (Color figure in appendix)

(A)



(B)

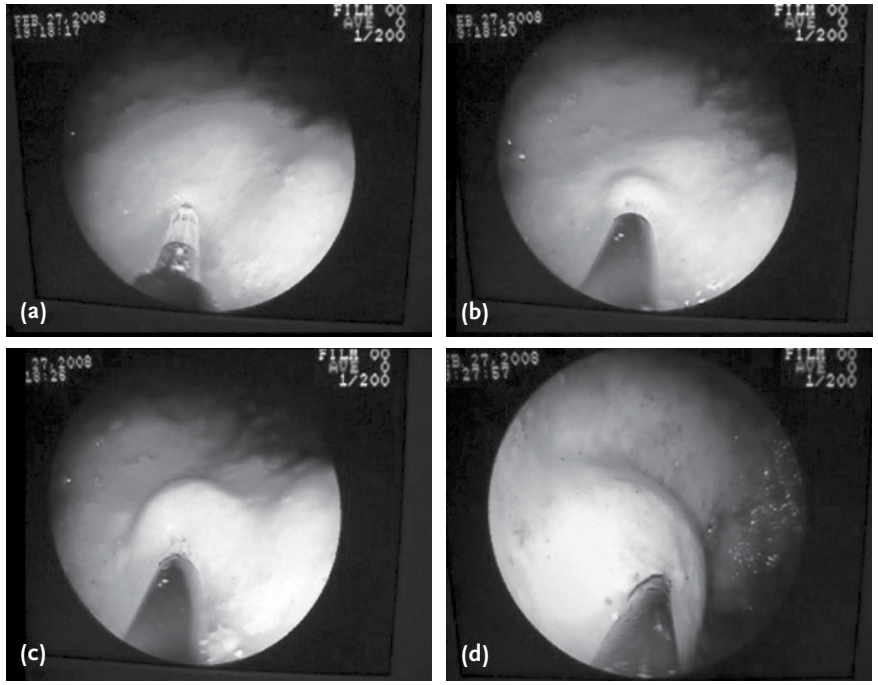


FIGURE 2: Endoscopic procedure of islet Tx into the GSMS (ENDO-STI). (A) Diagram of the procedure. (B) Endoscopic views during the procedure. (a) the endoscope needle is exposed, (b) inserted through the mucosa of the anterior gastric antrum at an approximate 45 degree angle, (c) the islets begin to be injected, and (d) the injection is completed. The procedure is then repeated at other sites. (Color figure in appendix)

the evening. Exogenous insulin was administered if the blood glucose exceeded 150mg/dL, and requirements were monitored twice daily. C-peptide levels were measured intermittently in some pigs by radioimmunoassay (Linco Research, St. Charles, MO), but IVGTTs were not carried out after islet Tx.

All recipients except one were followed for at least 21 days, with an elective maximum of 28 days (Table 2). When an infectious complication necessitated discontinuation of immunosuppressive therapy (Group 1, n=3), within a few days euthanasia was carried out before islet function was lost from acute cellular rejection.

NECROPSY AND HISTOLOGICAL EXAMINATION OF TRANSPLANTED ISLETS

Necropsy was performed in all pigs. Multiple biopsies of the pancreas, liver, and gastric wall were taken for microscopic examination for the presence of viable (insulin-positive) islets. Tissues were fixed in 10% buffered formalin, and paraffin sections were stained with H&E, polyclonal pig anti-swine insulin antibodies (Dako Cytomation, Carpinteria, CA), and rabbit anti-swine glucagon antibodies (Biogenex, San Ramon, CA), using standard procedures. Immunofluorescence studies for apoptosis were carried out. Specimens were fixed in 4% paraformaldeyde for histology. Frozen sections (10μm) were cut for the TdT-mediated dUTP-biotin nick-end labeling technique (TUNEL) and immunofluorescence analysis, and images were captured under a Nikon confocal microscope (Nikon D-Eclipse E800, Tokyo, Japan) using Nikon EZ-Cl (version 2.20) software. The TUNEL assay (ApopTag Fluorescein In Situ Apoptosis Detection Kit, Chemicon International, Temecula, CA) was carried out following the manufacturer's instructions. For insulin staining, the primary antibody was a rabbit anti-insulin antibody (dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), and the secondary antibody was a goat anti-rabbit IgG (dilution 1:500; Alexa fluor 488, Molecular Probes, Eugene, OR).

BIOPSY OF ISLETS IN THE GSMS

In some cases in the present study, we marked the sites of islet Tx into the GSMS by the injection of sterile ink (Spot ink, GI Supply, Camp Hill, PA), but this dispersed over a large surface area with time, and was found not to be helpful.

STATISTICAL ANALYSIS

Values are presented as mean±SEM. The statistical significance of differences was determined by Student t-test or by the non-parametric Wilcoxon-Mann-Whitney U test, where appropriate.

Table 2 Summary of individual experiments.

Pig ID numbers (R/D)	Strain (R+D)	Weight (kg) (R/D)	Gender (R/D)	IEQ/kg	Mean insulin dose before Tx (IU/kg/day)	Mean Insulin dose after Tx - day 20 (IU/kg/day)	Mean Insulin dose after Tx - last day (IU/kg/day)	Number of days without insulin	Survival (days)	Complications / Comments	Immuno-suppressive regimen (dose reduction)
Group 1A											
35907 / 37007	LLW	13.7 / >150	F/F	14,548	1.1	1.0	1.0	0	21	Low blood levels of MMF Euthanized	None
35807 / 37007	LLW	14.5 / >150	F/F	13,793	1.1	0.3	0.5	0	23	Low blood levels of MMF Euthanized	None
00108 / 01808	LLW	13.4 / >150	F/F	10,869	1.3	0	0	6	22	Bacteremia/ pneumonia Died	50%
03408 / 05208	LLW	12.9 / >150	F/F	25,523	1.8	0	0	7	21	Diarrhea Wound Infection Hypoglycemia* Euthanized	FK 75% MMF 100%
Group 1B											
00208 / 01808	LLW	17.0 / >150	F/F	4,116	1.4	NA	0	1	1	Elective euthanasia	None
39407 / 02408	LLW	11.4 / >150	F/F	6,150	1.4	NA	0	1	1	Elective euthanasia	None
03308 / 05108	LLW	12.6 / > 150	F/F	26,548	1.3	NA	0	4	14	Diarrhea Colitis/ bacteremia Intussusception Hypoglycemia* Euthanized	FK 50% MMF 100%
07908 / 09908	LLW	18.0 / >150	F/F	16,660	1.3	0.8	0	6	28	Diarrhea Pneumonia	FK50% MMF 100%
08008 / 09408	LLW	17.0 / >150	F/F	19,125	1.3	0.3	0	3	28	Diarrhea IV catheter infection Hypoglycemia*	50%
12208 / 14708	LLW	25.0 / >150	F/F	14,584	1.4	0	0	6	21	Diarrhea Bacteremia/ pneumonia Hypoglycemia* Died	50%
23808 / 23708	Yucatan	10.7 / 70	F/F	14,500	1.0	0	0	7	23	Lymphocytopenia Hypoglycemia* Died	50%
23908 / 23708	Yucatan	9.0 / 70	F/F	14,500	1.1	0.2	0.7	0	28	Food in stomach Technical problem during injection of islets Inadequate islet TX	None
Group 2											
325 / 389	LLW	16.0 / >180	F/F	10,000	1.2	0.8	1.2	0	28	None	None
23608 / 23408	Yucatan	13.4 / 75	M/F	15,125	1.6	1.8	1.7	0	28	None	None
23508 / 23408	Yucatan	13.2 / 75	M/F	15,000	1.5	2.5	2.1	0	28	None	None

*D = donor; LLW = Landrace large white; R = recipient; Tx = islet transplantation; Yucatan = miniature swine; FK = Tacrolimus; NA = not applicable. * Hypoglycemic episodes = glucose level <50mg/dL.*

Results

IN VITRO AND *IN VIVO* ASSESSMENT OF DONOR ISLET PREPARATIONS

The results of viability and functional assessment of donor islet preparations in Groups 1 and 2 are documented in Table 3. No statistically significant differences in viability, *in vitro*, or *in vivo* function could be determined between the groups.

FEATURES OF IBMIR

Minimal features of a pathologic nonspecific inflammatory response leading to islet disruption (e.g., rapid fall in blood glucose and rise in C-peptide) were seen in Group 1 (peak C-peptide 15min after Tx was a mean of 0.88±0.58ng/mL, compared with 0.15ng/mL pre-Tx), but features of the IBMIR were clearly seen in Group 2 (peak C-peptide at 15min 4.48±2.67ng/mL, compared with 0.11±0.05ng/mL pre-Tx) (Group 1 vs. 2 at 15 and 60min: $p<0.005$) (Figure 3).

EFFECT OF ISLET TRANSPLANTATION ON GLUCOSE METABOLISM

Group 1

Two Group 1 pigs were euthanized 24h after Tx for microscopic examination of the stomach wall. Within this time period, blood glucose levels were reduced; there was no further follow-up. Of the remaining pigs in Group 1 (n=10), one

Table 3 Results of *in vitro* viability and functional testing of donor islets.*

Group	# Pig donors	Post-culture recovery (%)	Viability (%)	Stimulation index (high glucose/basal)	Stimulation index (theophylline +high glucose/basal)
1A	n=3	80±2.9	82±1.7	2.5±0.60	9.9±2.15
1B	n=7	82±2.8	87±2.1	2.5±0.29	7.7±0.08
2	n=2	85/80	85/80	3.3/1.9	4.7/4.7

**There were no statistically significant differences in any parameters between the three groups (non-parametric Wilcoxon-Mann-Whitney U test). However, the statistical power of the test was low in view of the small number of samples in Group 2.*

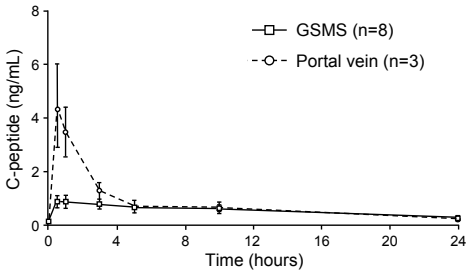


FIGURE 3: Serum C- peptide levels (mean±SEM) during the first 24h in pigs that received islet Tx either into the GSMS (Group 1, n=8) or portal vein (Group 2, n=3). In Group 1 there was a small increase in C-peptide within 15min after islet injection (reaching a mean of <1ng/mL), indicating that very few of the infused islets had been disrupted. In Group 2, the mean peak level at 15min was almost 4.5ng/mL, suggesting significant loss of islets from IBMIR. The differences in C-peptide at 15 and 60min between Groups 1 and 2 were statistically significant (both p<0.005).

pig showed no significant improvement in blood glucose levels or reduction in exogenous insulin requirement during 28 days of follow-up. At necropsy, only one site of insulin-positive islets could be confirmed histologically, suggesting that the injection of islets into other sites within the GSMS might have been unsuccessful. At the time of endoscopy, the stomach of this pig contained considerable food, making injection of the islets less certain than in the other cases. Peak C-peptide 15min after islet Tx (0.16ng/mL) was the lowest of all the Group 1 experiments at that time point (the second lowest being 0.28ng/mL and the mean and median being 0.88±0.58ng/mL and 0.9ng/mL, respectively, suggesting that fewer islets had been successfully transplanted.

In the remaining Group 1 pigs (n=9), the blood glucose level fell from a mean of 301±88mg/dL pre-Tx to 146±68mg/dL over the course of 20 days (p<0.001 vs. pre-Tx), the final level at the time of euthanasia or death (on days 21-28) being a mean of 125±62mg/dL (p<0.001 vs. pre-Tx) (Figure 4A). In some pigs (n=7) normoglycemia was maintained for several days without the need for exogenous insulin.

Exogenous insulin requirements were also significantly reduced from a mean of 1.30±0.30 IU/kg/day pre-Tx to 0.23±0.30 IU/kg/day on day 20 post-Tx (p<0.001 vs. pre-Tx), the final daily requirement at the time of euthanasia being a mean of 0.22±0.37 IU/kg/day (p<0.001 vs. pre-Tx) (Figure 4C). No differences were noted in the results between pigs in Group 1A and 1B.

Five Group 1 pigs developed episodes of hypoglycemia (blood glucose <50mg/dL) when insulin-independent that required i.v. glucose infusion. These episodes occurred in one pig as early as day 14 and in the others between days 21-25. Four of these died suddenly, and one required euthanasia for weight loss and infection. In 4 of the 5 cases, including 3 that died, hypoglycemic episodes were associated with a documented infectious complication (pneumonia with or without endocarditis/peritonitis). The fourth death was the result

of intussusception with colonic mucosal necrosis. Although there was no statistically significant correlation between the number of islets transplanted and the development of hypoglycemia, it may be of relevance that the 5 Group 1 pigs in which hypoglycemia developed received a mean of 20,056±2,585 IEQ/kg, whereas their 5 Group 1 non-hypoglycemic counterparts received a mean of only 14,074±933 IEQ/kg (p=0.06). (In comparison, the 3 Group 2 pigs received a mean of 13,333±2,886 IEQ/kg.) In some cases we were able to measure pig insulin and C-peptide levels during an episode of hypoglycemia, and these were found to be within the normal ranges for a non-fasting pig (not shown).

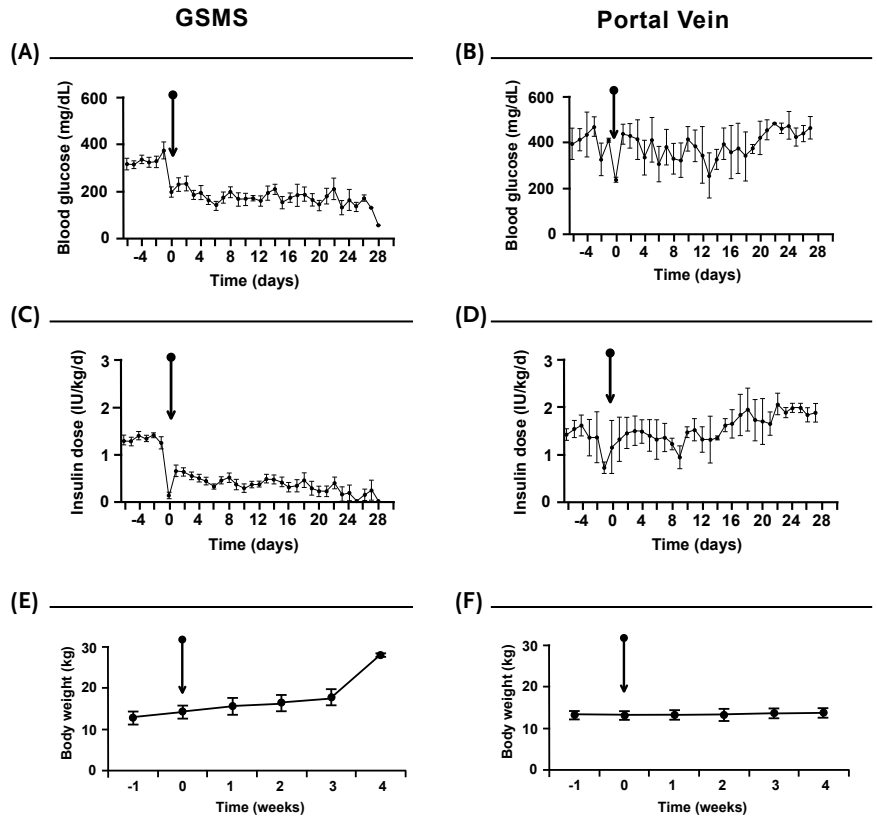


FIGURE 4: (A) Daily blood glucose levels (mean+SEM), (C) daily exogenous insulin requirements (mean+SEM), and (E) recipient weight (mean+SEM) in pigs that received islet Tx into the GSMS (Group 1, n=9). (B) Daily blood glucose levels (mean+SEM), (D) daily exogenous insulin requirements (mean+SEM), and (F) recipient weight (mean+SEM) in pigs that received islet Tx into the portal vein (Group 2, n=3). The arrows indicate the day of islet Tx. The increase in weight in the Group 1 pigs during the fourth week (E) is likely related to the fact that only 2 pigs remained in the study at 28 days. The relative lack of increase in weight in the Group 2 pigs (F) is likely related to the fact that 2 of the 3 pigs were Yucatan miniature swine, which gain weight more slowly than Landrace large white pigs.

Group 2

In Group 2, mean daily blood glucose levels did not change significantly over the 28-day period of follow-up (339±119mg/dL pre-Tx vs. 425±127mg/dL at day 20 and 466±51mg/dL at the time of euthanasia; p>0.05) (Figure 4B). There was no significant change in insulin requirement (1.43± 0.20 IU/kg/day pre-Tx vs. 1.71±0.83 IU/kg/day at day 20 and 1.66±0.45 IU/kg/day at the time of euthanasia; p>0.05) (Figure 4D).

CHANGES IN C-PEPTIDE LEVELS

Before STZ, there was no significant difference in mean fasting C-peptide level between healthy (non-diabetic) Landrace-large white crossbreed pigs (mean 0.34±0.18ng/mL) and Yucatan miniature swine (mean 0.36±0.14ng/mL). The response to intravenous glucose challenge, however, was greater in the Yucatan pigs. After STZ, fasting C-peptide levels were means of 0.11±0.02ng/mL (Landrace) and 0.14±0.04ng/mL (Yucatan), respectively (p>0.05), and the mean of all pigs was 0.12±0.03ng/mL. The mean post-STZ value (for the combined Groups 1 and 2) was significantly lower than the mean pre-STZ value (p<0.01), and there was no response at the IVGTT. Islet Tx was always between donor and recipient of the same breed. The mean post-Tx C-peptide value (0.27±0.20ng/mL) was significantly higher than the pre-Tx value (0.12±0.03ng/mL) (p<0.01), but fasting levels were again higher in Yucatan swine (in both Groups 1 and 2), though these did not correlate with blood glucose level or insulin requirement.

COMPLICATIONS OF IMMUNOSUPPRESSIVE THERAPY

When pigs were not specific pathogen-free, tacrolimus and MMF therapy was associated with diarrhea (necessitating a reduction or discontinuation of MMF) and/or systemic infection associated with pathogenic microorganisms, e.g., cryptosporidiosis, present when the pigs were admitted to our facility. The presence of infection necessitated discontinuation of immunosuppressive therapy and early euthanasia on humane grounds. When miniature swine were obtained from a specific pathogen-free herd, diarrhea and infection were not seen.

NECROPSY AND HISTOLOGICAL EXAMINATION OF TRANSPLANTED ISLETS

At necropsy, the native pancreas showed no or minimal viable (insulin-positive) beta cells in both Groups 1 and 2 (not shown).

The two Group 1 pigs that were euthanized 24h after Tx showed multiple insulin-positive islets (not shown). One Group 1 pig showed viable islets at only one site (as mentioned above). In the remaining Group 1 pigs (n=9), the GSMS showed evidence of a large number of viable islets at multiple sites

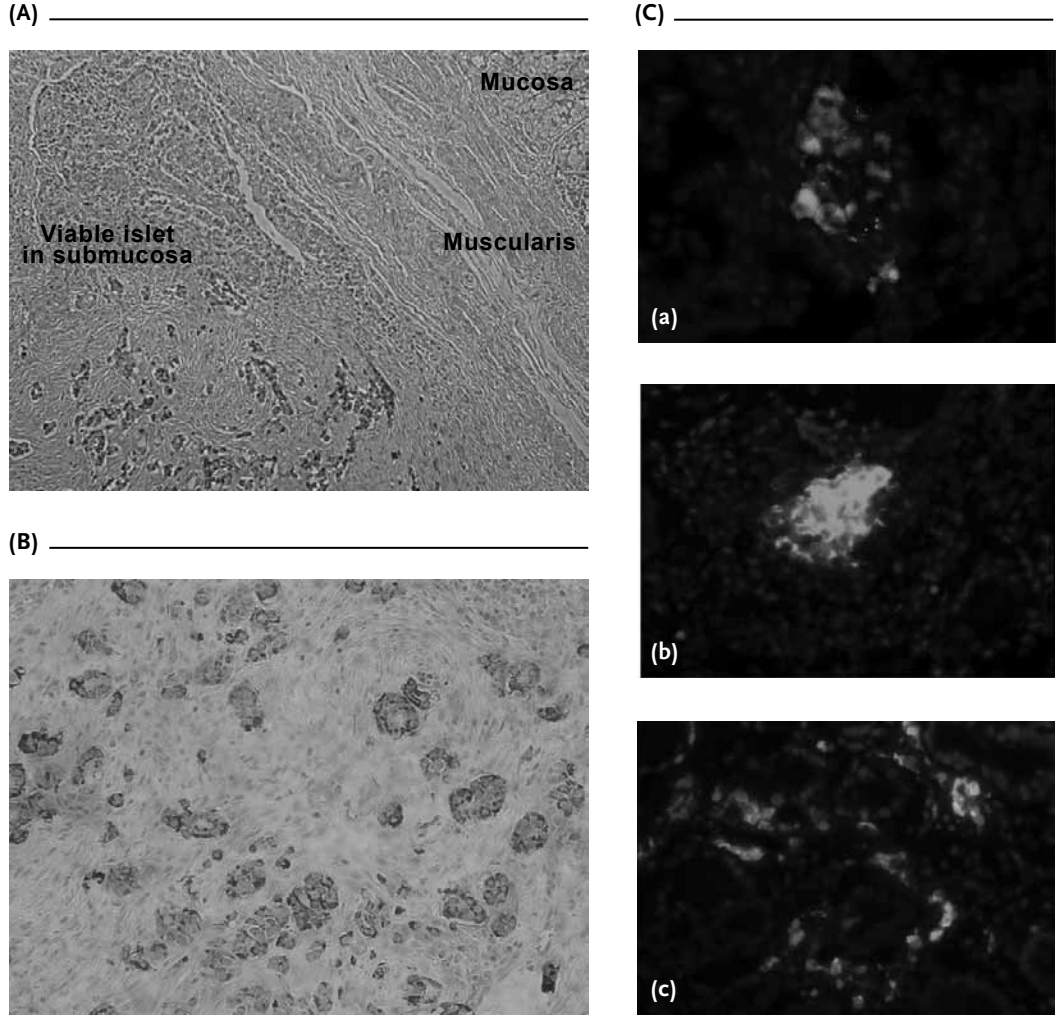


FIGURE 5: Histological appearances of the gastric wall following islet Tx into the GSMS in the Group 1 pigs. (A) Mucosa (upper right), muscularis mucosa, and GSMS (lower left), with viable insulin-staining of multiple islets (lower left) on day 14 after islet Tx (Pig #03308; H&E and insulin immunohistochemistry, magnification x10). (B) Healthy-looking islets 28 days after Tx, with a mild mononuclear cell infiltrate (Pig #07908; H&E and Insulin immunohistochemistry, x20). (C) Immunofluorescence staining for presence of apoptosis (red - TUNEL; green - insulin). (a) No TUNEL-staining was seen 24h after islet Tx, indicating an absence of apoptosis (Pig #00208). Late in the post-Tx period, e.g., 21-28 days, the incidence of apoptosis varied, being absent in some cases (b) (Pig #03408), but present in others (c) (Pig #35907). (Color figure in appendix)

(Figure 5A). A mononuclear cell infiltrate was seen around the islets when MMF or all immunosuppressive therapy had been discontinued a few days before euthanasia (Figure 5B).

Apoptosis was investigated by the TUNEL assay (Figure 5C). No apoptosis of islets was detected in the GSMS of the two pigs euthanized after 24h. At necropsy after 21-28 days, apoptosis was seen in some cases but not in others. Our impression was that there may have been greater apoptosis where (i) there were histological features suggesting some immunologic injury to the islets, (ii) the mononuclear cell infiltrate was more intense, and (iii) there were signs of inflammation, e.g., with positive staining for TNF-α (not shown). However, there was some variation, and no firm conclusions could be drawn.

In Group 2, extensive microscopic examination of multiple sections of the livers revealed no insulin-positive cells; although in one case there was one islet that stained weakly for glucagon, but not for insulin (not shown).

Discussion

The primary aims of this pilot study were to determine that (i) islets could be successfully transplanted into the GSMS, particularly by endoscopy, (ii) there would be no or minimal immediate loss of islets associated with a nonspecific inflammatory reaction, and (iii) they would function and survive. Our data indicate that all aims were achieved. Furthermore, the results were markedly superior to Tx into the portal vein.

In Group 1, both transplant techniques into the GSMS proved satisfactory, but endoscopy was simpler and quicker. There was one pig in which islet Tx was not entirely successful after ENDO-STI. If this pig is excluded, no difference in islet graft function or survival could be determined between the two approaches, and numerous insulin-positive cells were present microscopically in the islets in all cases even in the presence of a significant mononuclear cell infiltrate. We conclude that, from a technical perspective, the GSMS is a suitable site for islet Tx.

In contrast, although the number of experiments was small, islet Tx into the portal vein (Group 2) was followed by a uniform lack of success. This outcome was perhaps surprising in view of the relative success of islet Tx into the portal vein in humans and NHPs. However, to our knowledge, there are only two previous reports of islet Tx into the portal vein in pigs (23, 24). When pigs received a comparable immunosuppressive regimen to that used in the present study, success was limited unless sequential transplants were carried out, providing a total of a large numbers of islets (e.g., >20,000 IEQ/kg). It may be, therefore, that pig islet alloTx is a more difficult model. Nevertheless, in view of its anatomical similarities to humans and its size, we believe the pig provided an adequate model for this study, the main aims of which were to develop the endoscopic technique of islet Tx into the GSMS and to determine whether engraftment occurred.

Monitoring of peak C-peptide levels during the first 60min after islet Tx (as an indicator of disruption of islets) demonstrated a minimal loss of islets after Tx into the GSMS (Group 1) in contrast to the major loss from IBMIR seen after Tx into the portal vein (Group 2). Furthermore, the peak C-peptide seen in Group 1 was much lower than that reported in the literature after islet Tx into the portal vein (12). In addition, islets were not lost from apoptosis in the Group 1 pigs early (24h) after Tx.

The GSMS, therefore, has two important advantages over the portal vein; (i) It avoids IBMIR that results in loss of an estimated 60-80% of transplanted islets within the first few hours (9-12), and (ii) it should avoid the complications that are associated with portal vein injection of islets when performed by a percutaneous transhepatic catheter technique (2). However, a weakness of our study is our failure to perform IVGTTs or other similar tests, e.g., arginine stimulation test, during the post-Tx period. The results of these would have provided a better indication of the ability of the islets to respond to a glucose challenge.

The episodes of hypoglycemia documented in the post-Tx course in 5 pigs in Group 1 cannot be fully explained, but clearly require further investigation. Several possible causative factors can be considered. (i) Four of the 5 pigs had a serious infectious complication, although we would have anticipated that this might have been associated with hyperglycemia rather than hypoglycemia. (ii) All 5 pigs were anorexic at the time, and inadequate food intake could have been a factor, though this may imply that the islets were not responding normally to low blood glucose levels. (iii) Direct absorption of glucose through the gastric mucosa or muscular contraction of the stomach wall might stimulate islet activity and excessive insulin release, but this would likely have occurred in all pigs in Group 1. (iv) As very few islets were lost immediately after Tx, the number of islets transplanted may have been excessive. For example, the 5 pigs in which hypoglycemic episodes were documented received a mean of >20,000 IEQ/kg whereas their Group 1 non-hypoglycemic counterparts received little more than 14,000 IEQ/kg (p=0.06); an excessive number of islets might have played a role. In comparison, the Group 2 pigs received a mean of <13,500 IEQ/kg and may have lost approximately 66% from IBMIR; <4,500 IEQ/kg would have survived. The difference in islet mass between those pigs that developed hypoglycemia and those in Group 2 is therefore likely to have been considerable. If the large number of islets that survived in this Group 1 subset is a factor, this would again imply that insulin production did not respond to low glucose levels. This would seem unlikely, although it is known that intra-hepatic islets do not respond normally to a hypoglycemic event (25, 26). Our observations, though limited in number, that insulin and C-peptide levels remained within the normal ranges for a non-fasting pig during the episodes of hypoglycemia would also suggest that insulin production was not responding to the low level of blood glucose.

One further factor may need to be considered, namely that (v) In every case, islets from a large adult pig were transplanted into a young (2-3 months-old) piglet; studies at our center (27) have demonstrated that, in response to glucose challenge, the C-peptide peak at 15min is greater in adult pigs (2.13±0.25ng/mL) than in younger pigs (0.96±0.08ng/mL), and this vigorous response may have contributed towards hypoglycemia. In this respect, Service et al. (28) described a condition that is now recognized in the field of bariatric surgery in which 6 patients with type 2 diabetes had undergone gastric

bypass procedures for morbid obesity. When weight loss was significant, they developed hypoglycemic episodes (post-prandial neuroglycopenia). This was believed to be a nesidioblastosis-like syndrome in which possible hyper-function of pancreatic cells occurs, with over-production of beta cell tropic factors, such as GLP-1, that stimulate the islets to release excessive insulin. In our study, we used obese adult retired-breeder pigs as donors and transplanted the islets into juvenile pigs (of approximately one-tenth of the donor weight), and this could possibly have contributed towards the hypoglycemic episodes. We may have created a nesidioblastosis-like syndrome.

Following islet Tx into the portal vein, it is not possible to monitor for rejection by biopsy of the islets, as these are diffusely spread throughout the liver. In contrast, the GSMS is accessible to biopsy. However, a method of identifying the sites of islet Tx will be essential. Echo-endoscopy (endoscopic ultrasonographically-guided fine-needle aspiration) (29), although complex, might allow identification and biopsy of the islets in the GSMS.

Worldwide, millions of patients with diabetes might benefit from islet Tx. In the US alone, there are currently an estimated >2 million with T1D, and the number of patients developing T1D each year is estimated to be more >250,000 worldwide (30-32). The encouraging results that have been achieved to date following islet Tx into the portal vein, and the distinct possibility of islet xenoTx using genetically-engineered pig islets in the near future (33, 34), indicate that islet Tx may become a very active field of clinical therapy within a few years.

The availability of a simple, minimally-invasive and safe technical method of transplanting islets would prove a major step forward, particularly as it would allow repeated islet Tx, as is currently frequently required, with minimal stress and discomfort to the patient. Endoscopic delivery of the islets into the GSMS may fulfill this requirement, and has an additional advantage over the portal vein route in that it requires less equipment, and therefore should be less expensive.

However, although ENDO-STI appears to be safe from a technical perspective, the high incidence of hypoglycemic episodes seen in the present study is a matter of concern and requires investigation. A more comprehensive study in a healthier herd of pigs or in a NHP model, with a longer period of follow-up, is required before ENDO-STI can be considered equal or preferable to the intraportal route.

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CHAPTER 7

Investigation of lymphocyte depletion and repopulation using alemtuzumab (Campath-1H) in cynomolgus monkeys

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ABSTRACT

As the target CD52 molecule is expressed on erythrocytes of most NHP strains, using alemtuzumab in these species would cause massive hemolysis. Six cynomolgus monkeys of Indonesian origin, screened by agglutination assay for absence of CD52 on erythrocytes, were administered alemtuzumab in a cumulative dose to a maximum of 60mg/kg. In 2 monkeys, MMF was added as maintenance therapy. Complete depletion of T and B lymphocytes (>99.5%) was achieved with 20mg/kg alemtuzumab and was more profound than in monkeys treated with ATG (n=5), as quantified by flow cytometry. Repopulation was suppressed by weekly injections of 10mg/kg. Without MMF, repopulation of $CD20^+$ B cells and $CD8^+$ T cells was complete within 2 and 3 months, respectively, and repopulation of $CD4^+$ T cells was 67% after 1 year. MMF significantly delayed $CD4^+$ T cell repopulation. Among repopulating $CD4^+$ and $CD8^+$ T cells, a phenotypic shift was observed from $CD45RA^{hi}CD62L^{hi}$ naïve cells toward $CD45RA^{lo}CD62L^{lo}$ effector memory cells. In lymph nodes, the depletion of naïve cells was more profound than of memory cells, which may have initiated a proliferation of memory cells. This model offers opportunities to investigate lymphocyte depletion/repopulation phenomena, as well as the efficacy of alemtuzumab in preclinical Tx models.

Introduction

Alemtuzumab (Campath-1H) is a humanized anti-CD52 mAb that is approved for the treatment of lymphoid malignancies (1). Over the last decade, the off-label use of alemtuzumab as induction therapy for organ Tx has significantly increased. In transplant recipients, the profound and prolonged lymphocyte depletion following one or two doses has allowed for excellent graft survival with reduced maintenance immunosuppression (2-5). Longer-term follow-up should enable clarification as to whether this initial profound depletion will lead to a reduction in complications associated with chronic immunosuppressive drug use (6).

Preclinical studies, i.e., in NHP models, would be desirable to better understand the effects of alemtuzumab on the immune system and to determine whether it facilitates Tx tolerance or immunosuppression withdrawal. Using alemtuzumab in NHPs, however, has been hindered by the expression of the CD52 antigen on red blood cells (RBC) as well as on the targeted white blood cells (WBC) of most Old World monkeys (7,8). Treatment of such animals would result in lethal acute hemolytic anemia. Nevertheless, the successful administration of anti-CD52 antibodies in cynomolgus monkeys (*Macaca fascicularis*), negative for CD52 expression on RBC, has been reported (8).

The present study aimed at the establishment of a reliable screening assay for CD52 expression on RBC of cynomolgus monkeys. Once RBC-CD52-negative animals had been identified, a dose-efficacy study was undertaken to establish a NHP model for the use of alemtuzumab as induction therapy for experimental Tx. The effect of alemtuzumab on lymphocyte depletion was compared to that of ATG, and repopulation patterns were studied.

Materials & Methods

SOURCES OF BLOOD SAMPLES AND MONKEYS

Human blood was drawn from healthy volunteers after informed consent, as approved by the University of Pittsburgh Institutional Review Board (IRB#0608179). Blood was drawn from healthy baboons (*Papio anubis*, Oklahoma University Health Sciences Center, Oklahoma City, OK) and from cynomolgus monkeys (*Macaca fascicularis*, Three Springs Scientific, Perkasie, PA; Alpha Genesis, Yemassee, SC; and Primate Products, Miami, FL). All animal care procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985), and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

AGGLUTINATION ASSAY TO SCREEN FOR CD52 EXPRESSION ON RED BLOOD CELLS

An agglutination assay was used as described by De Giorgi et al. (9), with some modifications. Briefly, whole blood was washed 3 times in PBS. WBC were removed after each wash, leaving packed RBC that were resuspended in PBS to obtain a 1% suspension. In 96-well round-bottom plates, 50µL of the RBC suspension was incubated with 50µL of alemtuzumab (50µg/mL) (Campath 1-H, Genzyme, Cambridge, MA) for 10min at room temperature. 50µL mouse anti-human IgG (Coombs reagent, Immucor, Houston, TX) was added to facilitate agglutination. Wells with RBC and Coombs reagent only (but no alemtuzumab) were included as controls. Human RBC served as a negative control for CD52 expression, and baboon RBC as a positive control. After incubation for 1h at 37°C, the plate was centrifuged at 1500rpm for 1min. Agglutination was assessed macroscopically. A total of 2 humans, 3 baboons, and 38 cynomolgus monkeys were screened.

CONFIRMATION OF AGGLUTINATION WITH IMMUNOFLUORESCENCE

The RBC suspension was smeared onto a gelatin-coated microscope slide. Cells were fixed in 2% paraformaldehyde for 2-5min, and incubated with normal 20% goat serum for 1h at room temperature. Slides were incubated overnight with rat anti-human CD52 IgM (1:20; Serotec, Raleigh, NC) at 4°C. The following day, slides were incubated with Cy3-conjugated goat anti-rat IgM (1:500; Jackson ImmunoResearch, West Grove, PA) for 1h at room temperature. Photographs of fluorescence were taken through a Nikon Eclipse E800 microscope (Nikon Instruments, Melville, NY).

ANTIBODIES USED IN FLOW CYTOMETRY STUDIES

The following conjugated antibodies were used in flow cytometry experiments: anti-CD3 APC-Cy7 (clone: SP34-2), anti-CD4 FITC (M-T477), anti-CD8 PE-Cy7 (RPA-T8), anti-CD20 APC (2H7), anti-CD25 PE-Cy7 (M-A251), anti-CD45 PerCp-Cy5.5 (TU116), anti-CD45RA PE-Cy5 (5H9), anti-CD56 PE (MY31), anti-CD62L PE (SK11), anti-CD127 PE (hIL-7R-M21) (all from BD Biosciences, San Jose, CA), and anti-FoxP3 APC (PCH101) (eBioscience, San Diego, CA). A customized FITC-conjugated goat anti-human IgG (Zymed Labs, South San Francisco, CA) was used to detect the binding of alemtuzumab.

THE AFFINITY OF ALEMTUZUMAB FOR CYNOMOLGUS MONKEY LYMPHOCYTES *IN VITRO*

The human CD52 antigen is a 12 amino acid peptide, bound to the cell membrane by a glycosylphosphatidylinositol lipid anchor (10). Cynomolgus monkey CD52 has (only) 85% homology with human CD52 (11,12), resulting in a lower affinity of alemtuzumab for monkey CD52 (13), and a higher dose might be expected to be required to achieve comparable lymphocyte depletion. We compared the affinity of alemtuzumab for human, baboon, and cynomolgus monkey lymphocytes using flow cytometry. After lysis of RBC, WBC were incubated with alemtuzumab at 20, 10, and 2µg/mL for 30min. Cells were incubated with FITC-conjugated goat anti-human IgG, and antibodies against CD45, CD3, CD8, CD20, and CD56. (In cynomolgus monkeys, CD56 is expressed on monocytes rather than on natural killer (NK) cells [14]. NK cells are represented by $CD3^{-}CD8^{+}$ cells within the lymphocyte gate [15]). Cells were analyzed with a LSRII or FACS Aria II multicolor flow cytometer (BD). Data were analyzed using FacsDiva 6.0 software (BD).

ADMINISTRATION OF ALEMTUZUMAB OR ANTI-THYMOCYTE GLOBULIN TO CYNOMOLGUS MONKEYS

Six RBC-CD52-negative cynomolgus monkeys were selected. For blood withdrawal and i.v. alemtuzumab administration, catheters were inserted into the carotid artery and jugular vein, and led through a tether and jacket system to the exterior of the animal cage (16). The animals remained with catheters for a maximum period of 7 weeks.

Table 1 Alemtuzumab and ATG administration regimens (in mg/kg).

Alemtuzumab							
Monkey #	Day					Cumulative	Maintenance
	0	7	14	21	28		
M173-08	20					20mg/kg	
M170-08	20	20				40mg/kg	
M171-08	20	20	20			60mg/kg	
M175-08	20	10	10	10	10	60mg/kg	
M172-08	20	10	10	10		50mg/kg	MMF
M180-08	20	10	10	10		50mg/kg	MMF
ATG							
Monkey #	Day					Cumulative	Maintenance
	0	2					
M6075	17.4					17.4mg/kg	MMF, anti-CD154
M4154	6.4	6.4				12.8mg/kg	MMF, anti-CD154
M7355	8.6	8.6				17.2mg/kg	MMF, anti-CD154
M174-08	6.8	6.8				13.5mg/kg	MMF, anti-CD154
M122-08	8.9	8.9				17.9mg/kg	MMF, anti-CD154

MMF = mycophenolate mofetil

In clinical practice, transplant patients are treated with one or two doses of 30mg alemtuzumab, resulting in a cumulative dose of approximately 0.5-1.5mg/kg. Based on the literature and our *in vitro* studies, we estimated the minimum efficacious dose in monkeys to be 20mg/kg/body weight.

Prior to each alemtuzumab or ATG infusion, metaclopramide (0.5mg/kg), diphenhydramine (5mg/kg), methylprednisolone (5mg/kg), and aminopentamide (0.05mg) were administered i.v. to prevent the adverse effects of the 'cytokine storm' that can occur. Monkeys were treated with a cumulative dose of a maximum of 60mg/kg alemtuzumab (Table 1). Alemtuzumab was infused over a 4h time-period. In 2 monkeys, immunosuppression was maintained by oral MMF at 50-100mg/kg/day to maintain trough levels of 3-5µg/mL. Monkeys treated with 13-18mg/kg ATG (n=5) in our preclinical Tx models were used as historical controls to compare the extent of lymphocyte depletion (Table 1) (17). Ganciclovir (5mg/kg once daily) was administered for as long as lymphopenia (<500 leukocytes/µL) persisted, to prevent viral reactivation. Serum samples, available in 5 of 6 monkeys, from before and 4-6 weeks after treatment were tested for CMV reactivation by qualitative PCR (Zoologix, Chatsworth, CA).

THE EFFICACY OF ALEMTUZUMAB ASSESSED BY FLOW CYTOMETRY

Before treatment, whole blood was incubated with antibodies against CD45, CD3, CD4, CD8, CD20, and CD56, after which RBC were lysed. Flow cytometry was performed to determine baseline numbers of T cells, B cells, monocytes, and NK cells. Absolute cell numbers were calculated based on the WBC counts obtained from our institution's hematology laboratory. Numbers of regulatory T cells (T_{Reg}) were analyzed using anti-CD25, anti-CD127, and anti-FoxP3 antibodies. Intracellular staining for FoxP3 was performed according to the manufacturer's protocol (eBioscience). In 4 monkeys, T cell subsets were analyzed using antibodies against CD45RA and CD62L to define naïve cells (T_N) ($CD45RA^{hi}CD62L^{hi}$), effector memory cells (T_{EM}) ($CD45RA^{lo}CD62L^{lo}$), terminally differentiated effector memory cells (T_{EMRA}) ($CD45RA^{hi}CD62L^{lo}$), and central memory cells (T_{CM}) ($CD45RA^{lo}CD62L^{hi}$) (18).

Flow cytometry was carried out 2h after the first infusion of alemtuzumab, and then at least 3 times weekly for two weeks, followed by weekly and later monthly monitoring. Depletion and repopulation of lymphocytes after alemtuzumab were compared to cynomolgus monkeys treated with ATG.

MONITORING OF LYMPHOCYTE DEPLETION IN LYMPH NODES BY FLOW CYTOMETRY AND HISTOLOGY

After ketamine sedation and local anesthesia, lymph nodes from the inguinal or axillary regions were excised (i) before the administration of alemtuzumab in all 6 monkeys, and (ii) 15 days (n=6), (iii) 31 days (n=3), and (iv) 1 year (n=4) after the first infusion, to determine the extent of lymphocyte depletion in secondary lymphoid tissues. Immediately after collection, approximately half of the tissue was fixed in 10% formalin or 4% paraformaldehyde for histologic examination. The other half was weighed, and cells were obtained, counted, and analyzed by flow cytometry. Formalin-fixed tissue was stained for H&E. Cryo-sections of paraformaldehyde-fixed tissue were cut and stained for immunofluorescence for the presence and density of $CD3^+$ and $CD20^+$ cells.

STATISTICAL ANALYSES

Continuous variables are expressed as mean±SD, and compared using the Student t-test. p-values <0.05 were considered to indicate a statistically significant difference. All analyses were performed with GraphPad Prism 4.

Results

SCREENING FOR CD52 EXPRESSION ON RED BLOOD CELLS

At the described concentrations, alemtuzumab and Coombs reagent consistently agglutinated baboon RBC, whereas they did not agglutinate human RBC. Agglutination was readily observed macroscopically (Figure 1A). Control wells with human, baboon or cynomolgus RBC and Coombs reagent only (in absence of alemtuzumab) never agglutinated. Thirty-eight cynomolgus monkeys, originating from Chinese (n=12), Cambodian (n=12), and Indonesian (n=14) breeding colonies, were screened. RBC from Chinese and Cambodian monkeys were uniformly agglutinated by alemtuzumab (24 of 24), whereas RBC from monkeys of Indonesian origin (region of Djakarta, island of Java) consistently tested negative (14 of 14) (Figure 1A). Agglutination was either clearly positive or clearly negative; non-interpretable or intermediate levels of agglutination were not observed. Immunofluorescence confirmed the results of the agglutination assay in 100% of the monkeys (Figure 1B).

THE AFFINITY OF ALEMTUZUMAB FOR CYNOMOLGUS MONKEY WHITE BLOOD CELLS

The affinity of alemtuzumab for cynomolgus monkey T and B cells was substantially lower than for the human equivalents (Table 2). The highest concentration of alemtuzumab used for cynomolgus cells (20µg/mL) still resulted in a markedly lower mean fluorescent intensity (MFI) than the lowest concentration (2µg/mL) used for human cells. For human cells, higher concentrations of alemtuzumab proved directly toxic, probably after cross-linking with the secondary antibody, as previously described (19). With cynomolgus monkey cells, this phenomenon did not occur, also indicating that the binding of alemtuzumab to monkey CD52 is suboptimal. Based on these data we hypothesized that the minimum efficacious dose in cynomolgus monkeys would be 20mg/kg. Alemtuzumab did not bind significantly to monocytes and granulocytes (not shown) of either cynomolgus monkeys or humans.

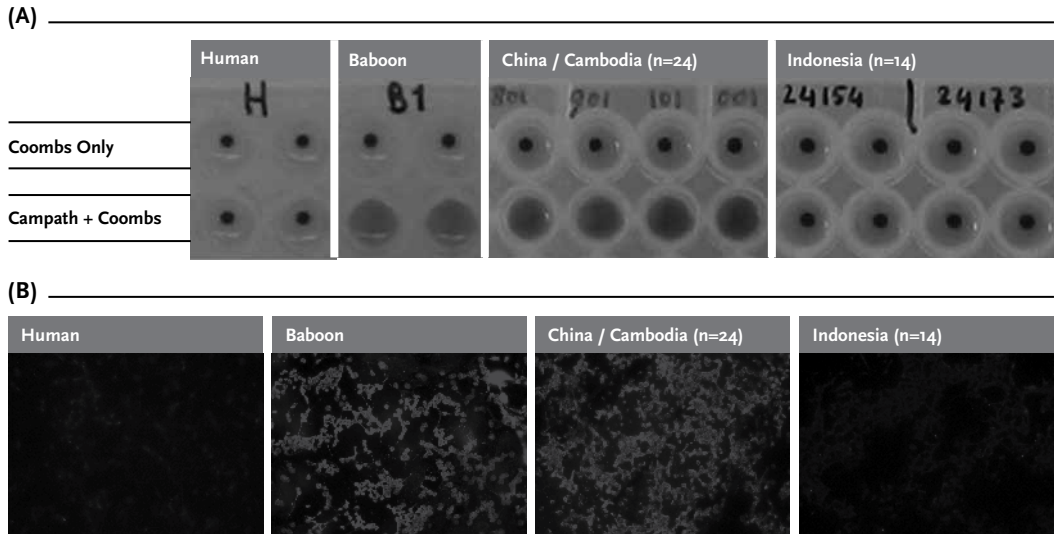


FIGURE 1 | CD52 EXPRESSION ON RED BLOOD CELLS (RBC): (A) Representative results of the agglutination screening test. Non-agglutinated RBC were spun towards one point in the round-bottom wells. In contrast, wells with agglutinated cells had a cloudy appearance as a result of the RBC being fixed to one another by alemtuzumab and secondary antibody. (B) Immunofluorescent staining of RBC confirmed the agglutination results, showing bright staining for CD52 on all baboon and Chinese and Cambodian monkey RBC. Staining was completely absent on human and Indonesian monkey RBC. (Color figure in appendix)

MINIMAL ADVERSE EFFECTS OF ALEMTUZUMAB ADMINISTRATION

After pre-treatment with metaclopramide, diphenhydramine, methylprednisolone, and aminopentamide, the infusion of 10 or 20mg/kg of alemtuzumab over a 4h time-period was uncomplicated in 19 of 19 infusions. No decrease in blood pressure was observed, indicating that the 'cytokine storm' was well-controlled by the pre-treatment agents. The hematocrit was 35.8±2.4% before alemtuzumab treatment, 33.3±3.0% after 14 days, and 38.8±3.7% after 60 days (both not significantly different from pre-treatment). Liver and kidney function was unaltered (not shown). During profound lymphocyte depletion, the monkeys remained healthy with no signs of infection. Two monkeys developed a slowly progressive neutropenia ($<1.0x10^9$ cells/L) after they had been treated with cumulative doses of 50 and 60mg/kg alemtuzumab, respectively, which was readily corrected by a single subcutaneous injection of human granulocyte colony-stimulating factor (G-CSF 5µg/kg; Neupogen, Amgen, Thousand Oaks, CA). Even though the number of $CD8^+$ T cells recovered rapidly with a high peak in 2 monkeys (Figure 2B) (which could have been a sign of viral infection), no clinical features of infectious disease were observed, and PCR for CMV was negative in all tested monkeys after 4-6 weeks of lymphopenia.

Table 2 Affinity of alemtuzumab for white blood cell subsets of cynomolgus monkey, baboon, and human.

	Cells	Concentration		
		20µg/mL	10µg/mL	2µg/mL
Cyno – Indonesia	$CD4^+$ T	4.58	3.37	1.71
	$CD8^+$ T	2.00	1.41	1.11
	$CD20^+$ B	1.49	1.30	1.20
	$CD56^+$ (monocytes)	0.60	0.61	1.08
Cyno – China/Cambodia	$CD4^+$ T	6.01	5.59	2.30
	$CD8^+$ T	2.20	2.37	1.63
	$CD20^+$ B	2.89	2.71	1.81
	$CD56^+$ (monocytes)	0.97	1.22	1.39
Baboon	$CD4^+$ T	0.91	0.83	0.76
	$CD8^+$ T	0.76	0.79	0.83
	$CD20^+$ B	2.37	2.01	0.98
	$CD56^+$ (monocytes)	0.63	0.58	0.55
Human	$CD4^+$ T			201
	$CD8^+$ T			88.6
	$CD20^+$ B			14.0
	$CD56^+$ (monocytes)			4.02

The affinity of alemtuzumab is expressed as relative mean intensive fluorescence (MFI), which was calculated as the ratio of (i) the MFI of the cell subset stained with alemtuzumab and secondary antibody and (ii) the MFI of the background fluorescence of the same cell subset incubated with the secondary antibody only (when alemtuzumab was omitted). Cyno = cynomolgus monkey

EFFICACY OF ALEMTUZUMAB IN DEPLETING LYMPHOCYTES

After the initial dose of 20mg/kg, nearly total depletion of $CD4^+$ and $CD8^+$ T cells, B cells, and NK cells was achieved in all 6 monkeys (Figure 2). Significant depletion could be observed as early as 2h after the infusion was completed and was maximal after 4 days, with $CD4^+$ and $CD8^+$ T cell counts <3 cells/µL. In comparison with high-dose ATG, depletion of T cells after alemtuzumab was significantly greater ($CD4^+$: 99.9±0.07% vs. 95.0±4.83%, p=0.03; $CD8^+$: 99.9±0.25% vs. 95.0±3.32%, p=0.005) (Figure 2A and B). In addition, alemtuzumab almost totally depleted $CD20^+$ B cells, whereas the effect of ATG on B cells was less profound ($CD20^+$: 99.6±0.40% vs. 72.3±22.2% depletion, p=0.01) (Figure 2C). The effect of both agents on monocytes ($CD56^+$, Figure 2D) was variable. Both agents depleted NK cells, with alemtuzumab having a marginally greater effect (Figure 2E).

REPOPULATION OF LYMPHOCYTES

Seven days after the initial dose of alemtuzumab, the beginning of repopulation could be observed, i.e., the mean number of $CD4^+$ T cells had risen to 14 cells/µL (Figure 2A). This repopulation was suppressed by subsequent weekly doses of alemtuzumab and resulted in maintenance of T cell numbers of <50cells/µL for a maximum period of 32 days (Figure 2A and B). The infusion of 10mg/

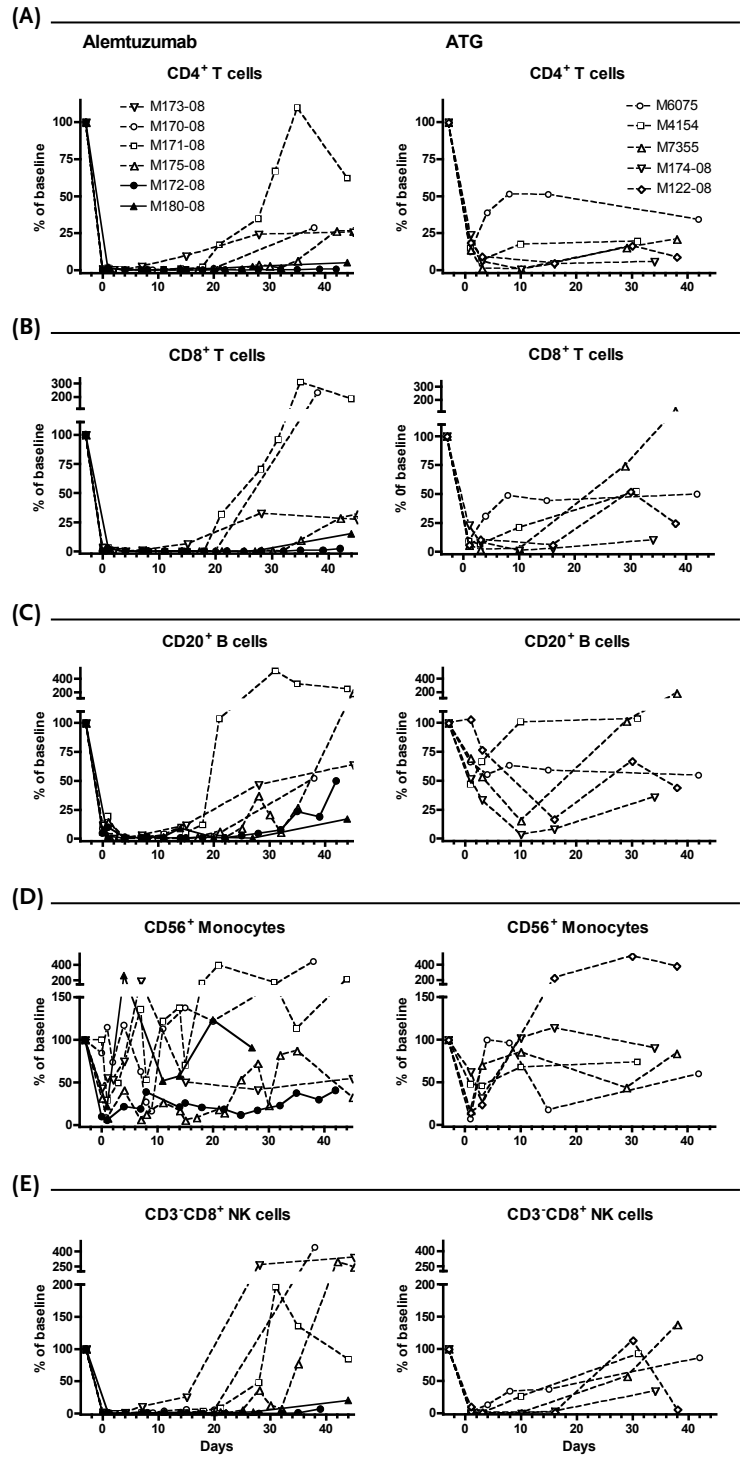


FIGURE 2 | DEPLETION OF LYMPHOCYTES AFTER THE *IN VIVO* ADMINISTRATION OF ALEMTUZUMAB OR ATG IN CYNOMOLGUS MONKEYS: $CD4^+$ and $CD8^+$ T cells (A and B), $CD20^+$ B cells (C) and $CD3^-CD8^+$ natural killer (NK) cells (E) were almost totally depleted after 20mg/kg alemtuzumab (left panels). Depletion was more profound than with ATG (right panels). No clear effect on the number of $CD56^+$ monocytes was observed (D).

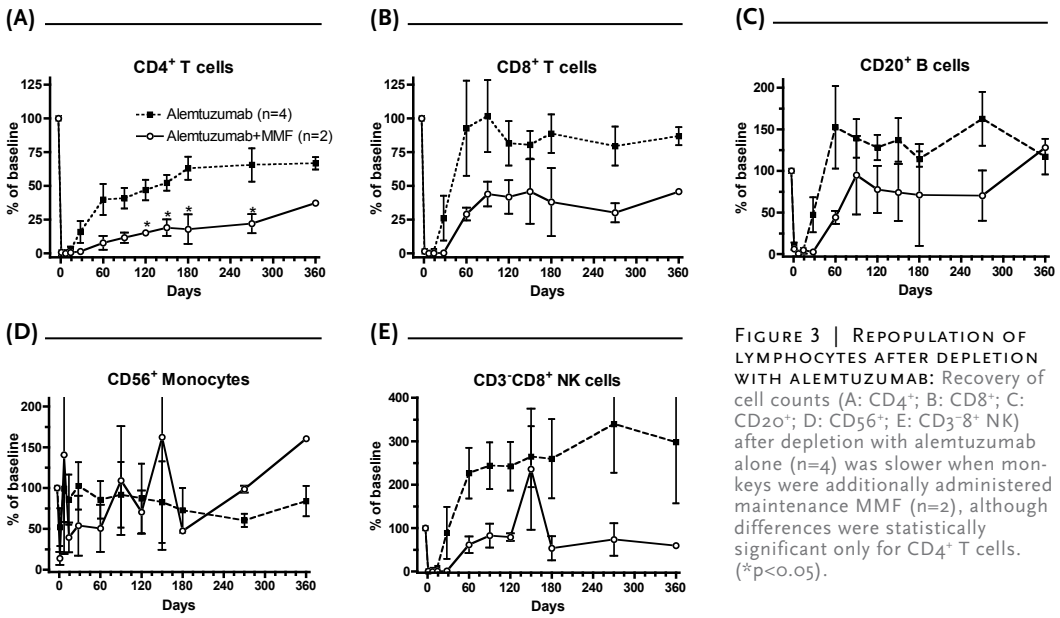


FIGURE 3 | REPOPULATION OF LYMPHOCYTES AFTER DEPLETION WITH ALEMTUZUMAB: Recovery of cell counts (A: $CD4^+$; B: $CD8^+$; C: $CD20^+$; D: $CD56^+$; E: $CD3^-8^+$ NK) after depletion with alemtuzumab alone (n=4) was slower when monkeys were additionally administered maintenance MMF (n=2), although differences were statistically significant only for $CD4^+$ T cells. (*$p<0.05$).

kg at weekly intervals appeared to be sufficient to maintain this lymphopenia. However, after each subsequent dose of alemtuzumab, cell repopulation occurred more rapidly within the following 7 days. In monkey M175-08, the fifth dose on day 28 was no longer able to fully deplete the T and B cells.

Although short-term depletion and recovery patterns differed with different alemtuzumab treatment schedules (1 to 5 doses), repopulation seemed to follow a similar pattern after 45 days (Figure 3). Without MMF, after 1 year of follow-up, $CD4^+$ T cells had recovered up to 67±9.3% of baseline numbers (Figure 3A). $CD8^+$ T (Figure 3B) and $CD20^+$ B cells (Figure 3C) reached 100% of baseline numbers within 3 and 2 months, respectively. Repopulation of $CD4^+$ T cells was significantly delayed by chronic MMF therapy (Figure 3A). However, for other cell subsets the observed differences were not statistically significant. (One animal has to date completed only 9 of 12 months follow-up).

A cell subtype that was seen increasingly in the blood during repopulation had a $CD3^-CD8^+$ phenotype (Figure 3E). These cells, seen in the lymphocyte gate on flow cytometric analysis, have been described to have functional characteristics of NK cells (15).

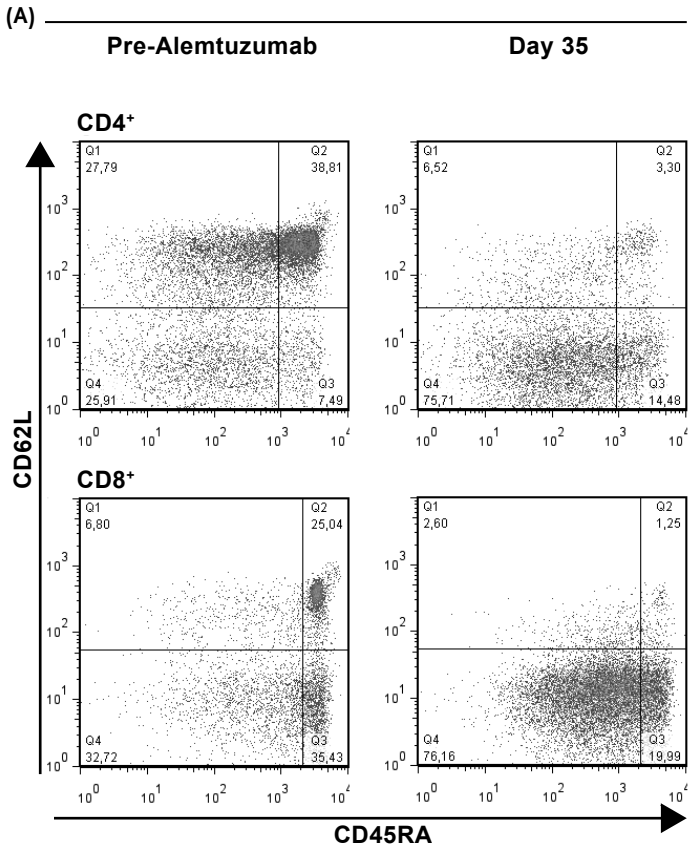
PHENOTYPE OF REPOPULATING T LYMPHOCYTES

Before the administration of alemtuzumab, a majority of $CD4^+$ and $CD8^+$ T cells possessed the phenotypic characteristics of T_N ($CD45RA^{hi}CD62L^{hi}$) (Figure 4A, left panels). In 4 monkeys (2 treated with alemtuzumab and 2 with alemtuzumab+MMF) the initial repopulating lymphocytes had a predominant T_{EM} phenotype ($CD45RA^{lo}CD62L^{lo}$) (Figure 4A, right panels, and Figure 4B, left panels). The percentages of naïve and memory cells seemed to return to pre-treatment levels after 180 days. In absolute cell numbers, the repopulation of T_{EM} cells occurred faster than of T_N. MMF treatment did not seem to alter this pattern (T_{EM} appearing first, Figure 4B). It should be noted that the small number of animals in each group did not allow further qualitative analyses. $CD8^+$ T cells with a memory phenotype reached numbers that were persistently higher than pre-treatment numbers.

The relative numbers of $CD4^+CD25^{hi}CD127^-FoxP3^+$ T_{Reg} before, and 2 and 4 months after alemtuzumab, were compared. No significant differences in percentages of T_{Reg} were observed; the percentage of T_{Reg} was <2% of total $CD4^+$ cells at all time-points (data not shown). Therefore, alemtuzumab did not seem to selectively spare T_{Reg} from depletion or promote their relative expansion after lymphocyte depletion.

LYMPHOCYTE DEPLETION IN PERIPHERAL LYMPH NODES

Before the administration of alemtuzumab, examination of lymph nodes stained with H&E revealed normal architecture, with B cell follicles being visible in the cortical region at low magnification. Lymph nodes examined after alemtuzumab administration showed no significant changes in this respect (not shown). However, when cell populations obtained from lymph nodes before and after alemtuzumab were compared by flow cytometry, a significant reduction in cell numbers was observed. Mean $CD4^+$ T cell numbers were reduced from 407±176x10^3 cells/mg of analyzed tissue before administration to 51±29x10^3 cells/mg at 15 days after alemtuzumab (87% depletion, n=6, p=0.0007). Mean $CD8^+$ T cell numbers were reduced from 210±136x10^3 to 16±10x10^3 cells/mg (92% depletion, n=6, p=0.0058), and $CD20^+$ B cells from 129±69x10^3 to 44±37x10^3 cells/mg (66% depletion, n=6, p=0.024). After subsequent alemtuzumab infusions, these cell counts were not reduced further in lymph nodes taken at day 31 (n=3). After 1 year of follow-up, cell counts had returned to baseline levels (n=4, without MMF) (data not shown). With immunofluorescence for $CD3^+$ and $CD20^+$ using confocal microscopy, major reductions in T and B cell densities were observed (not shown). Flow cytometric analysis of lymph node T cell subsets before and 15 days after alemtuzumab showed that T_{EM} were relatively resistant to depletion (Figure 5).



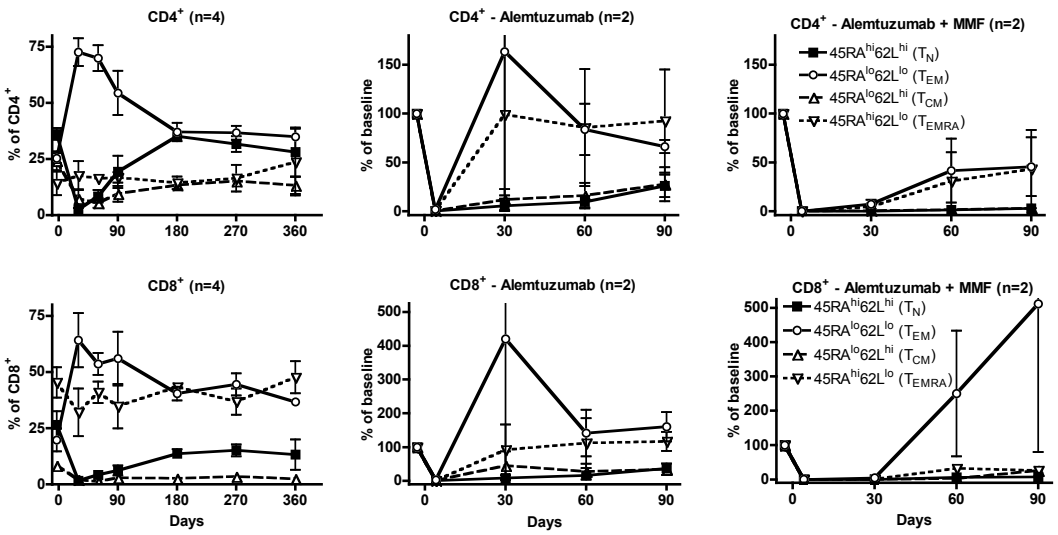


FIGURE 4 | DEPLETION-INDUCED HOMEOSTATIC PROLIFERATION OF MEMORY T CELLS:
(A) Compared to pretreatment levels, a shift from naïve T cells (T_N - $CD45RA^{hi}CD62L^{hi}$) toward effector memory T cells (T_{EM} - $CD45RA^{lo}CD62L^{lo}$) was observed, here shown for 1 representative monkey (M171-08). (B) Changes in the percentages of naïve and memory T cell subsets among $CD4^+$ and $CD8^+$ T cells (left panels). The addition of MMF did not alter its pattern (right vs. middle panels).

Discussion

Clinical experience with alemtuzumab indicates that there are unanswered immunological questions that require further investigation. A preclinical NHP model would prove useful for this purpose. This study proves the feasibility of using alemtuzumab in cynomolgus monkeys of Indonesian origin. The administration of alemtuzumab to NHPs for induction therapy has not been reported previously, probably because most NHPs express the target antigen, CD52, on their RBC as well as on their WBC. In the present study, all tested cynomolgus monkeys that originated from the island of Java, Indonesia, were RBC-CD52-negative. The same species from China or Cambodia were consistently positive for CD52 on RBC. This suggests a genetic diversity among cynomolgus monkeys, which could be geographically determined, or could result from inbreeding in established captive colonies. Using an agglutination screening method, animals could be selected for CD52-negativity on RBC, and thus severe hemolysis following administration of alemtuzumab could be avoided.

Once suitable monkeys were identified, the infusion of alemtuzumab was safe; no early or late side-effects or complications were observed. The efficacy of alemtuzumab at high dose was excellent, with almost complete depletion of all T and B lymphocytes in peripheral blood, and a near-complete depletion of cells in the peripheral lymph nodes. At the dosages used, in comparison with ATG, more profound T cell depletion was achieved. In addition, B cells were completely depleted from the blood, a result generally not achieved by ATG. B cell depletion may be important for long-term transplant survival. In cynomolgus monkeys it has been documented that islet allograft survival is enhanced when the repopulating B cells are maintained in an immature state by maintenance immunosuppression (20).

However, our experience of the relative effects of alemtuzumab and ATG must be viewed in the light of the dosages administered. Because of the proven lower affinity of alemtuzumab for monkey lymphocytes, a dose approximately 20 times higher than used in clinical practice was used. This dosage was compared with ATG given at the standard dosages we have used in NHP studies at our center (17, 21, 22). At clinical dosages, it is possible that the results may have been different.

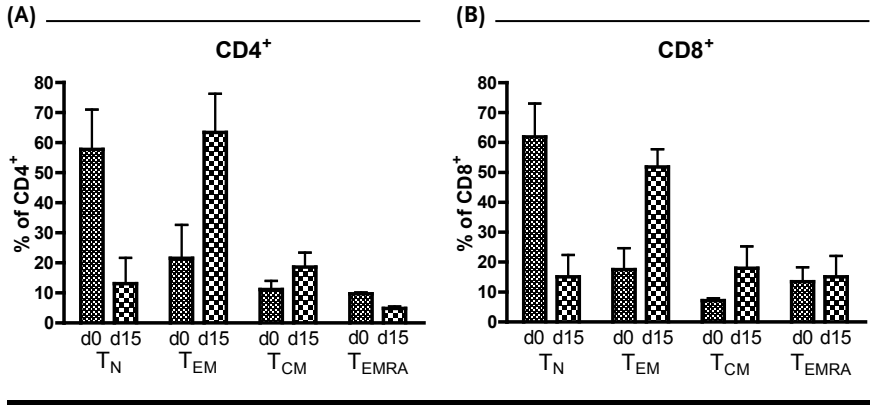


FIGURE 5 | LYMPHOCYTE DEPLETION IN PERIPHERAL LYMPH NODES: Changes in naïve and memory T cell subsets from lymph nodes of 3 monkeys. $CD4^+$ (A); $CD8^+$ (B). d0 = before alemtuzumab; d15 = 15 days after alemtuzumab. (T_N = naïve T cells; T_{EM} = effector memory T cells; T_{CM} = central memory T cells; T_{EMRA} = terminally-differentiated effector memory T cells).

In comparison to human transplant recipients, who are treated with 1 or 2 doses of alemtuzumab (usually at an absolute dosage of 30mg) (5), complete depletion of lymphocytes in cynomolgus monkeys was maintained for a shorter period of time. A number of reasons may account for this observed difference. First, although alemtuzumab has a half-life of >8 days (23,24), it is likely that the lower affinity of alemtuzumab for cynomolgus CD52 causes the mAb concentration to fall to a subtherapeutic level within a few days, leading to a quicker repopulation. Second, maintenance immunosuppression after alemtuzumab, as applied in clinical practice, contributes to the prevention of lymphocyte recovery (25). In two of our experiments, the addition of MMF delayed $CD4^+$ T cell recovery. MMF, an inhibitor of purine synthesis, is an immunosuppressive agent that directly affects the cell cycle in T and B cell proliferation. Third, in the absence of concomitant immunosuppression, the reduction in the efficacy of alemtuzumab after multiple dosing (with more rapid repopulation after each administration) could be due to neutralizing anti-alemtuzumab antibodies that the monkeys develop against the humanized IgG. We did not investigate this. An additional possibility could be a reduced expression of CD52 on the repopulating T_{EM}, which could be a subject for further studies.

After almost complete T cell depletion, an increase in T_{EM} was observed as an increased percentage of total T cells and in absolute numbers. It is possible that the relatively higher numbers of surviving T_{EM} in the peripheral lymph nodes (compared to T_N) initiated 'homeostatic repopulation' (26). On the other hand, expansion and conversion of T_N into T_{EM} may have occurred (27). The expansion of memory T cells after lymphocyte depletion has been observed in patients with organ transplants who have received alemtuzumab as induction therapy (28,29). Moreover, kidney transplant recipients treated with alemtuzumab and

deoxyspergualin monotherapy all experienced acute cellular rejection with T_{EM} predominating in blood samples and in graft histology (29).

Recent in vitro studies have shown that healthy individuals harbor T_{EM} subpopulations readily expressing IFN-γ and lytic molecules upon allostimulation, underscoring the potential threat these cells may pose to transplanted organs (18). These experiments support the hypothesis that the human alloresponse is not 'truly primary', but is a response mediated by both naïve and memory T cells (30,31). After depletion with alemtuzumab, expanded T_{EM} clones cross-reactive with graft antigens could theoretically cause acute cellular rejection without the need for antigen presentation and costimulation. Homeostatic proliferation has proven to be a barrier for tolerance induction in rodents (32).

At our institution, alemtuzumab induction has been used extensively in clinical Tx programs. Recently, the results following 3-year follow-up after living kidney Tx were published (5). Despite excellent results regarding patient and graft survival, in 25% of patients an episode of acute cellular rejection occurred, though this was generally very mild (Banff 1) and responsive to methylprednisolone. The incidence of acute cellular rejection increased sharply 1 year after alemtuzumab therapy. Weaning of tacrolimus monotherapy as per protocol, repopulation of lymphocytes in general, or homeostatic proliferation of T_{EM} (as observed in our experiments) could all have contributed to this increased late incidence.

Clinical implementation of innovative immunosuppressive therapies often requires prior testing in a NHP model. This will be particularly important with regard to the establishment of a clinically relevant immunosuppressive regimen in xenoTx. Profound lymphocyte depletion with alemtuzumab followed by relatively low-dose maintenance immunosuppressive therapy could perhaps facilitate a reduction in the relatively heavy drug regimens that have hitherto been required in xenoTx, bringing its clinical application a step closer.

In conclusion, the identification of cynomolgus monkeys of Indonesian origin to which alemtuzumab can be administered safely allows for a NHP model that can be utilized to clarify immunological questions relating to the above *in vitro*, pre-clinical, and clinical observations. In this model, the effects of induction therapy with alemtuzumab on the immune system can be explored, and the question of whether it facilitates the reduction of maintenance immunosuppressive therapy and/or the development of Tx tolerance can be investigated.

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FANTS IN CELLS

CHAPTER 8

T-lymphocyte homeostasis and function in infant baboons: implications for transplantation

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Submitted.

ABSTRACT

Laboratory mice are born lymphopenic and demonstrate lymphopenia-induced proliferation that generates memory T cells, yet they are prone to immunological tolerance. Here we tested whether these fundamental immunologic observations apply to higher animals by studying the immune system of infant baboons. Using flow cytometry of peripheral blood cells, it was found that baboons are born relatively lymphopenic and subsequently expand their initially naïve T cell pool with increasing numbers of memory T cells. After Tx of an artery patch allograft or xenograft, non-immunosuppressed recipients readily mounted an immune response against donor-type antigens, as evidenced by mixed lymphocyte reaction. Immunosuppression with ATG, anti-CD154 mAb and MMF prevented T cell-mediated rejection. After lymphocyte depletion with ATG, homeostatic T cell proliferation was observed. In conclusion, the baboon proved a suitable model to investigate the infant immune system. In the present study, neonatal lymphopenia and expansion of the memory T cell population were observed but, unlike mice, there were no indications that infant baboons are prone to T cell tolerance. The expansion of memory T cells during the neonatal period or after induction therapy may form an obstacle to tapering immunosuppressive therapy, or ultimately, achieving immunologic tolerance.

Introduction

Laboratory mice are born lymphopenic and demonstrate lymphopenia-induced proliferation (LIP), which generates memory T cells during the first few weeks of life (1). During the neonatal period, their T cell immune system exhibits remarkable plasticity and, although no longer believed to be immunodeficient, is prone to tolerance induction after Tx or adoptive transfer of splenocytes under certain conditions (reviewed in Adkins et al. [2]). Unlike rodent species, the knowledge available in humans indicates that human neonates are born with an almost entirely functional immune system, a difference believed to be influenced by the duration of gestation (3). However, also for humans there are several indications that the neonatal immune system still has immature features. For example, (i) the immune system of neonates is still antigen-inexperienced and is constituted by naïve T lymphocytes (4, 5). (ii) In the unfortunate event that an infant needs an organ transplant, the Tx of a heart across the ABO-blood group barrier is relatively uncomplicated (6-9), while in adults this may lead to graft loss from hyperacute rejection (10). Moreover, when an ABO-incompatible (AB-I) donor organ is introduced before the development of anti-AB antibodies, B cell tolerance to the incompatible blood group antigen(s) can develop (11). (iii) Pediatric or adult recipients of umbilical cord blood cells experience less graft-versus-host disease than recipients of bone marrow Tx from an adult donor (12, 13).

To our knowledge, few studies have closely followed the development of the human infant immune system with advancing age (14, 15), possibly due to ethical concerns of involving infants in research protocols. Alternatively, NHP models can be of value for investigation of the developing immune system (16).

The aim of this study was to verify two fundamental immunologic principles observed in mice, i.e., neonatal LIP of T cells and the possibility of inducing T cell tolerance to transplant antigens, in a NHP model using infant baboons. We found that baboon T lymphocytes significantly increase in number after birth with transient appearance of memory T cells. After alloTx or xenoTx of an artery patch graft (from AB-I baboon donors, or WT pig donors, respectively) at the age of 3 months, the T cell immune system appeared functional and, in the absence of immunosuppressive therapy (IS), indications for the allowance of immunologic tolerance were not observed.

Materials & Methods

SOURCES OF ANIMALS AND BLOOD SAMPLES

Healthy young infant baboons (*Papio anubis*) (Group 1, Table 1) were housed at the specific pathogen-free facility of the University of Oklahoma Health Sciences Center (UOHSC) (17). Blood was drawn from 6 baboons during the first week of life, and at 1, 2, 4, and 6 months of age, stored in tubes containing EDTA, and shipped at 4°C to the University of Pittsburgh (UPitt) for analysis on the following day. The infants remained healthy and untreated throughout the period of study. Blood samples from healthy, untreated, young baboons aged 1 year (n=6), and 2-3 years (n=6) were drawn and shipped under the same conditions.

In addition, 5 infant baboons (Group 2) were housed at UPitt from the age of 2 months for immunologic and Tx studies. Blood was drawn and analyzed before and at multiple time-points after Tx of an artery patch graft (see below).

All animal care procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1985), and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

TRANSPLANTATION OF BABOON OR PIG ARTERY GRAFT

At the age of 3 months, 5 baboons (Group 2) were transplanted with a length of donor baboon aorta or donor pig carotid artery, as an immunologic model for pediatric heart Tx. Donor artery grafts were obtained either from adult baboons (UOHSC) that were AB-I with the recipient, or from WT pigs (Large White/ Landrace) of blood type O (Country View Farms, Schellsburg, PA). Under full anesthesia, and after laparotomy and partial heparinization (100IU/kg), the recipient aorta was clamped distally to the renal arteries and at the bifurcation, incised longitudinally, and a 1.0x0.5cm patch of donor (baboon or pig) artery was sutured into the aortic wall as a full-thickness onlay graft. The clamps were removed and, after assuring hemostasis, the abdomen was closed.

Table 1 Experimental groups

Group	Experiment				
1	Non-transplanted, No IS	6 Baboons aged 0 to 6 months 6 Baboons aged 1 year 6 Baboons aged 2-3 years			
		Baboon #	Blood group	Graft type	Age at Tx (days)
2A	Transplanted, No IS	7707 7607	B B	A Pig	95 102
2B	Transplanted, IS	7507 5008 5508	B A B	A B Pig	98 107 98

IS = Immunosuppression

Table 2 Immunosuppressive and supportive therapy in Group 2 baboons.

Induction therapy	Dose	Duration
Thymoglobulin	2.0-2.5mg/kg i.v,	Days -3 and -1
Methylprednisolone	5mg/kg i.v.	Before each dose of ATG and on day 0. The dose was then reduced by 1mg/kg/d, and discontinued on day 5
Maintenance therapy	**Dose**	**Duration**
Anti-CD154 mAb	20-25mg/kg i.v.	Days -1, 0, 4, 7, 10, 14, then every 5-7d
Mycophenolate mofetil	20-150mg/kg/d p.o. divided in 2 doses	Begun on day -2 (to maintain a blood trough level of 3-6µg/ml)
Supportive Therapy	**Dose**	**Duration**
Cefazolin	25mg/kg bid i.v	For 3 days after surgery
Famotidine	0.25mg/kg bid i.v.	From day -3
Ganciclovir	5mg/kg i.v.	From day -4 until 1 month post-Tx
Ketorolac	0.5mg/kg i.v	Before every dose of anti-CD154 mAb
Buprenorphine	0.01mg/kg bid i.v.	For 3 days after surgery

Two baboons (Group 2A) received either an AB-I or WT pig graft, but no IS; these baboons were followed for immunologic studies for 6 weeks until euthanasia for pathologic studies. Three baboons (Group 2B) received an AB-I (n=2) or a WT pig graft (n=1), and were immunosuppressed throughout follow-up after Tx (Table 1). IS consisted of induction with ATG, and was maintained using an anti-CD154 mAb and MMF (Table 2). Group 2B baboons were followed for 15 weeks after Tx.

Table 3 Monoclonal antibodies used to define lymphocyte subsets by flow cytometric analysis.

	Fluorochrome-conjugated monoclonal antibodies						
Lymphocyte subset	Pacific blue	PE-Cy7	APC-Cy7	FITC	PE-Cy5	PE	APC
T and B cells	$CD3^+$	$CD4^+$	$CD8^+$	$CD20^+$			
Naïve T cells (T_N)	$CD3^+$	$CD4^+$	$CD8^+$		$CD45RA^{hi}$	$CD62L^{hi}$	
Total memory T cells (T_{TotMem})	$CD3^+$	$CD4^+$	$CD8^+$		$CD45RA^{lo}$		
Effector memory T cells (T_{EM})	$CD3^+$	$CD4^+$	$CD8^+$		$CD45RA^{lo}$	$CD62L^{lo}$	
Central memory T cells (T_{CM})	$CD3^+$	$CD4^+$	$CD8^+$		$CD45RA^{lo}$	$CD62L^{hi}$	
Terminally differentiated effector memory T cells (T_{EMRA})	$CD3^+$	$CD4^+$	$CD8^+$		$CD45RA^{hi}$	$CD62L^{lo}$	
Regulatory T cells (T_{Reg})	$CD3^+$	$CD4^+$	$CD25^{hi}$			$CD127^-$	$FoxP3^+$

FLOW CYTOMETRY STUDIES

The following fluorochrome-conjugated mAbs were used in flow cytometry experiments: anti-CD3 Pacific Blue (clone: SP34-2), anti-CD4 PE-Cy7 (SK3), anti-CD8 APC-Cy7 (RPA-T8), anti-CD20 FITC (2H7), anti-CD25 APC-Cy7 (M-A251), anti-CD45RA PE-Cy5 (5H9), anti-CD62L PE (SK11), anti-CD127 PE (hIL-7R-M21) (all from BD), and anti-FoxP3 APC (PCH101) (eBioscience).

Whole blood was incubated with conjugated antibodies or corresponding isotype controls, after which red blood cells were lysed using PharmLyse (BD). Intracellular staining for FoxP3 to identify regulatory T cells (T_{Reg}) was performed according to the manufacturer's protocol (eBioscience). Cells were analyzed with a LSRII multicolor flow cytometer (BD). Data were analyzed using FacsDiva 6.0 software (BD). Table 3 shows how different lymphocyte subsets were identified. Absolute cell numbers were calculated based on white blood cell counts obtained from our institution's hematology laboratory.

MIXED LYMPHOCYTE REACTION

Mixed lymphocyte reaction (MLR) measured by ^{3}H-thymidine incorporation was carried out as previously described (18). Briefly, as stimulator cells, PBMC were isolated from buffy coats of (i) 150ml of blood from an unrelated adult baboon (cryopreserved in aliquots to provide stimulator cells for each experiment) or (ii) from freshly obtained blood from Large White/Landrace WT pigs. As responder cells, PBMC obtained from buffy coats of fresh infant baboon blood were isolated. In the MLR, responder cells ($0.4x10^6$ cells/well) were stimulated with irradiated adult baboon or WT pig PBMC at a 1:1 ratio. All responder-stimulator combinations were set up in quadruplicate and were incubated for 5 days. Ten microliters of ^{3}H-thymidine labeling medium (1μCi/well; New England Nuclear, Boston, MA) were added to each well during the last 18 hours of incubation. Cells were harvested on glass-fiber filter mats with a cell harvester, and were analyzed by beta-scintillation counting on a liquid scintillation counter (PerkinElmer, Waltham, MA). The mean results of quadruplicate tests were expressed as counts per million and stimulation index (average counts of anti-baboon or anti-pig response divided by anti-self response).

GRAFT HISTOLOGY

At necropsy, lengths of aorta, including the graft, were fixed in 10% formalin and embedded in paraffin. Four-micrometer (4μm) sections were cut and stained with H&E for light microscopy.

STATISTICAL ANALYSES

Continuous variables are expressed as mean±SEM, and compared using the (paired) Student t-test, linear regression, and repeated measures ANOVA for changes over time, as appropriate. Repeated measures ANOVA was followed by pairwise comparisons of peak values with values at birth. p-values <0.05 were considered statistically significant. All analyses were performed with GraphPad Prism 4.

Results

EARLY LYMPHOCYTE DEVELOPMENT IN HEALTHY INFANT BABOONS

In Group 1 baboons aged 0 to 30 months, a linear decrease in the percentage of $CD4^+$ T lymphocytes ($CD4^+$ cells) was observed (Figure 1A). This decrease was complemented by a steady increase in the proportion of $CD8^+$ T lymphocytes ($CD8^+$ cells) (Figure 1A). During the first months of life, an increase in the absolute number of lymphocytes occurred. Numbers of $CD4^+$ cells were 2.8±0.8x higher at the age of 4 months compared with numbers at birth (repeated measures ANOVA, $p<0.0001$, Figure 1B). Numbers of $CD8^+$ cells were 6.6±1.4x higher at the age of 4 months compared with numbers at birth (repeated measures ANOVA, $p<0.0001$). Because of the changing proportions over time, the fold-increase in CD8+ cells was greater than of $CD4^+$ cells (t-test, $p=0.034$). After 4 months, a decline in CD4+ cells, and stabilization of the numbers of $CD8^+$ cells was observed. No distinctive pattern in the development of $CD20^+$ B lymphocytes (B cells) could be discerned (Figures 1C, 1D).

After observing the above trends in lymphocyte development early in life, we were interested in characterizing the phenotype of different T lymphocyte subpopulations (Figure 2A). In the first week of life, the majority of lymphocytes (62.1±7.1% of $CD4^+$, and 58.5±7.9% of $CD8^+$) possessed the phenotypic characteristics of naïve T cells (T_N), which stain positive for CD45RA and CD62L (Figures 2B, 2C). The absolute numbers of $CD4^+$ and $CD8^+$ T_N increased during the first 4 months of life (Figures 2D, 2E), indicating T cells leaving the thymus (new thymic emigrants). However, as a percentage of total $CD4^+$ and $CD8^+$ cells, a decline in T_N occurred during the first 2 months (repeated measures ANOVA, $p=0.015$ for $CD4^+$, $p=0.003$ for $CD8^+$, Figures 2B, 2C). Simultaneously, an increase in the percentages of effector memory T cells (T_{EM}) and central memory T cells (T_{CM}) was observed (in Figure 2 presented separately as well as combined as total memory T cells [T_{TotMem}]). The changes were most pronounced in the $CD8^+$ population. When compared for differences in kinetics, the appearance of T_{EM} occurred earlier than the appearance of T_{CM} (Figure 2C). Figures 2F and 2G show the development of T_N and T_{TotMem} as a ratio of their respective baseline numbers at birth (set as 1.0), indicating that the expansion of T_{TotMem} was more significant than of T_N (paired t-test, $p<0.05$ for most time-points). Beyond 2 months of age, the proportions of T_N and T_{TotMem} reversed back to proportions more comparable with those at birth. The percentages and numbers of terminally differentiated effector memory T cells (T_{EMRA}) remained relatively stable (not shown).

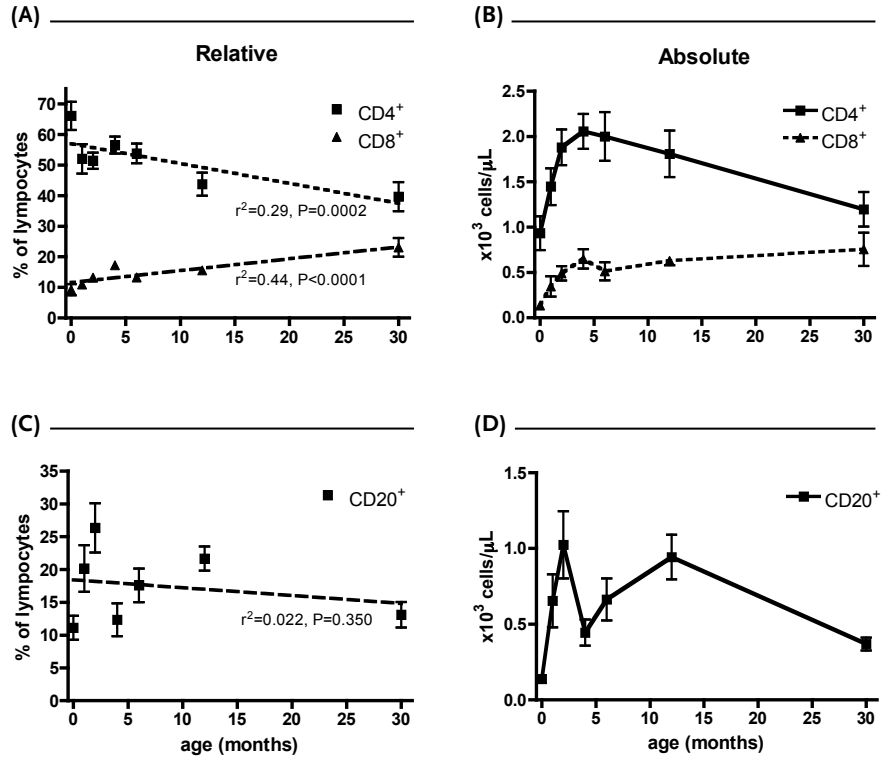


FIGURE 1 | CHANGES IN $CD4^+$ AND $CD8^+$ T CELLS, AND $CD20^+$ B CELLS IN HEALTHY INFANT BABOONS (GROUP 1): Left panels show proportions of $CD4^+$ (A) and $CD8^+$ (C) cells of total lymphocytes. Right panels show absolute numbers $CD4^+$ (B) and $CD8^+$ (D) cells, which increased 3 to 7 fold during the first 4 months of life.

The relative and absolute numbers of $CD25^{hi}FoxP3^+CD127^-$ T_{Reg} peaked at the age of 4 months (repeated measures ANOVA, both $p<0.0001$, Figure 3), at the same time-point as the highest measured total numbers of $CD4^+$ cells.

The dynamic rearrangements of T_N and T_{TotMem} did not seem to affect the functionality of the cellular immune system to mount an immune response, as investigated by MLR. The responses of infant baboon PBMC after stimulation with irradiated baboon or WT pig PBMC did not vary with age ($p>0.05$ for each), (Figure 4).

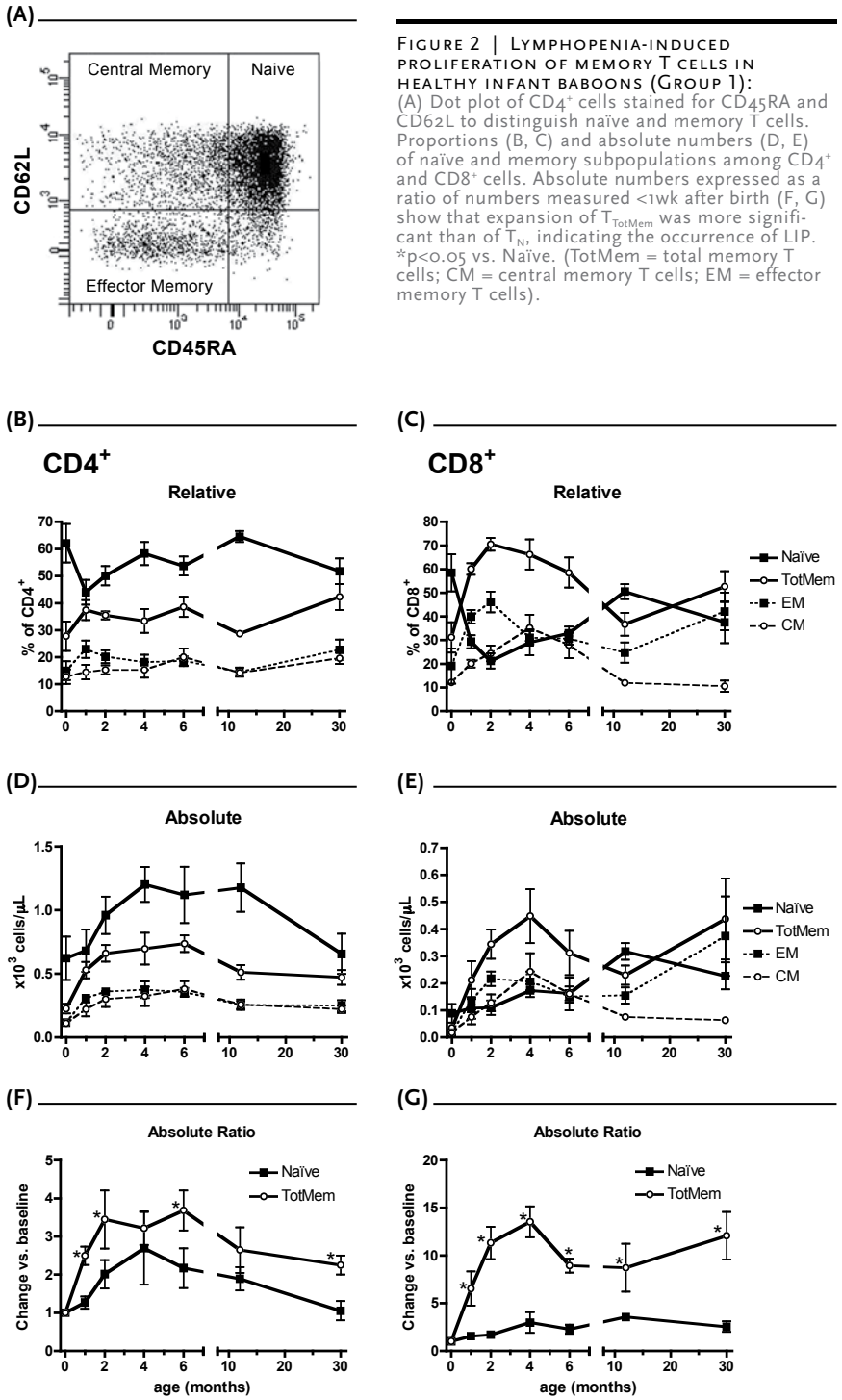


FIGURE 2 | LYMPHOPENIA-INDUCED PROLIFERATION OF MEMORY T CELLS IN HEALTHY INFANT BABOONS (GROUP 1): (A) Dot plot of $CD4^+$ cells stained for CD45RA and CD62L to distinguish naïve and memory T cells. Proportions (B, C) and absolute numbers (D, E) of naïve and memory subpopulations among $CD4^+$ and $CD8^+$ cells. Absolute numbers expressed as a ratio of numbers measured <1wk after birth (F, G) show that expansion of T_{TotMem} was more significant than of T_N, indicating the occurrence of LIP. *p<0.05 vs. Naïve. (TotMem = total memory T cells; CM = central memory T cells; EM = effector memory T cells).

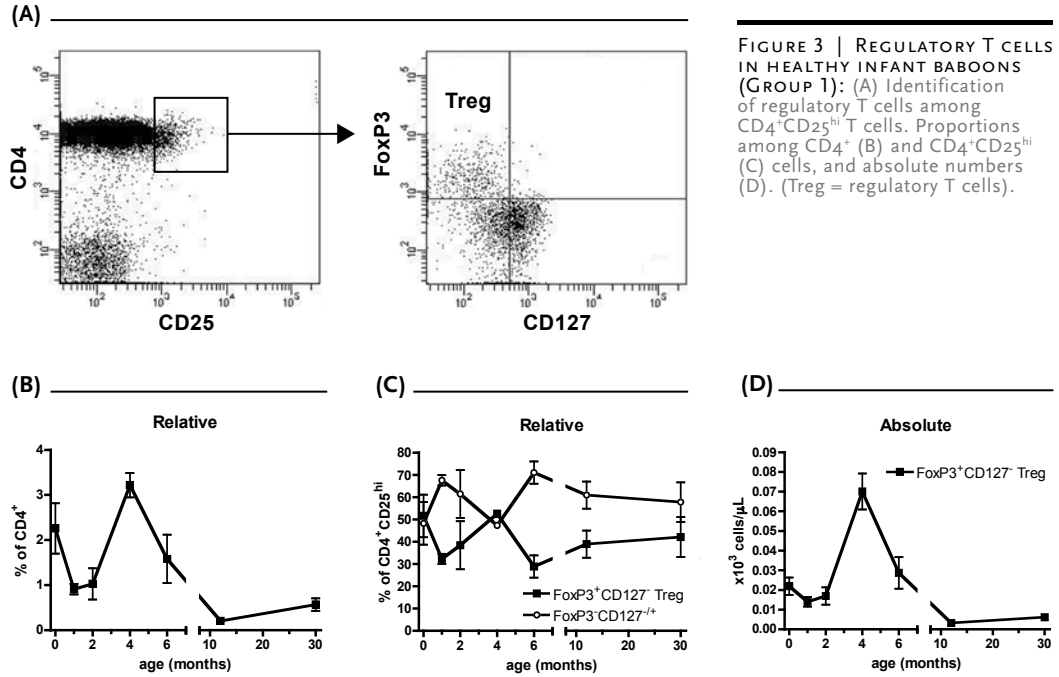


FIGURE 3 | REGULATORY T CELLS IN HEALTHY INFANT BABOONS (GROUP 1): (A) Identification of regulatory T cells among $CD4^+CD25^{hi}$ T cells. Proportions among $CD4^+$ (B) and $CD4^+CD25^{hi}$ (C) cells, and absolute numbers (D). (Treg = regulatory T cells).

CHANGES IN LYMPHOCYTE SUBPOPULATIONS AFTER ARTERY PATCH TRANSPLANTATION

Group 2A: transplantation in absence of IS

After Tx of an artery patch graft in 2 untreated infant baboons, the gradual decline in percentage of $CD4^+$ and increase of $CD8^+$ cells did not appear different from those in healthy untreated and non-transplanted baboons of comparable age (Figure 5A). The presence of an AB-I or WT pig graft therefore did not seem to influence this evolution.

Three weeks post-Tx, a slight and transient, and non-significant, shift to increased proportions of memory T cells (T_{TotMem}, T_{EM}, T_{CM}) was seen for $CD4^+$ as well as $CD8^+$ cells (repeated measures ANOVA, p=0.374 for $CD4^+$, Figure 6A, and p=0.243 for $CD8^+$, Figure 6B), without major changes in absolute numbers (Figure 6C, 6D).

After Tx without IS, no change in T_{Reg} as percentage of $CD4^+$ cells was detected (Figure 7A). However, although we were not able to provide statistical evidence due to the small number of baboons, within the $CD4^+CD25^{hi}$ population, greater numbers of cells expressed FoxP3 (Figure 7B), resulting in a peak of absolute numbers of T_{Reg} 4 weeks after Tx (Figure 7C).

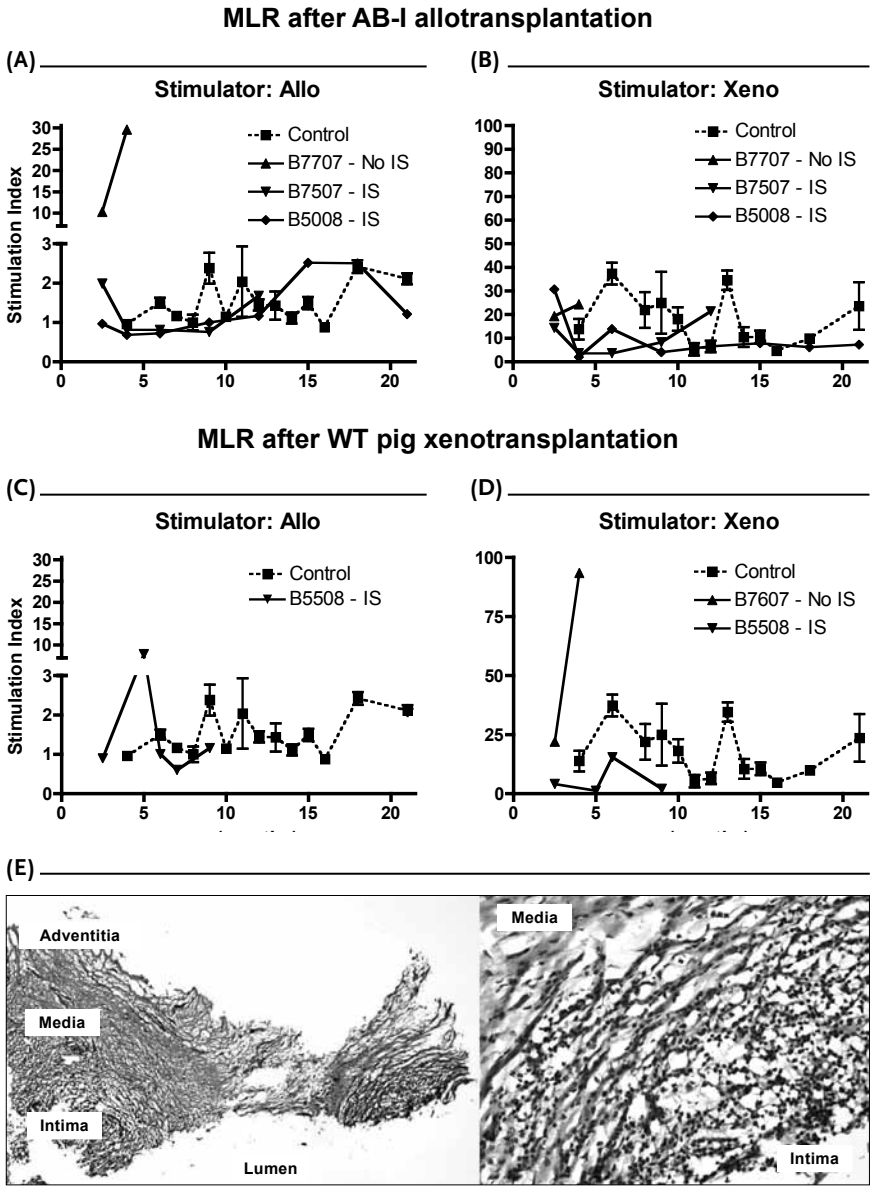


FIGURE 4 | STIMULATION INDEX AFTER MIXED LYMPHOCYTE REACTION (MLR) AND HISTOLOGY IN TRANSPLANTED (GROUP 2) BABOONS: Responses in healthy infant baboons (Control) are compared with responses in baboons after AB-I allo Tx (A, B) and WT pig xeno Tx (C, D) in the presence (IS) or absence (No IS) of immunosuppressive therapy. Data for B7607 after allo-stimulation are missing due to technical error (C). (E) Graft histology 6 weeks after Tx without IS (Group 2A). Left panel (magnified 4x) shows loss of architecture. The location of tunica media in the vessel wall can still be recognized by collagen and smooth muscle (pink), but vast cellular infiltration has caused damage throughout the vessel wall (B7607). Right panel: lymphocytic infiltration of intima and tunica media (magnified 40x, B7607). Clear (transparent) spaces are artifacts due to suboptimal tissue storage. (Color figure in appendix)

Although the changes in T_N and T_{TotMem} were small, during follow-up donor type-specific immunosensitization had readily occurred. This was evidenced by an increased response in the donor type-specific MLR (Figures 4A-D), the appearance of antibodies against graft antigens in recipient serum (anti-A antibodies in AB-I Tx, and anti-Gal antibodies in WT xenoTx, respectively, Dons EM et al. manuscript submitted), and fibrosis and lymphocyte infiltration of the graft on histological examination (Figure 4E).

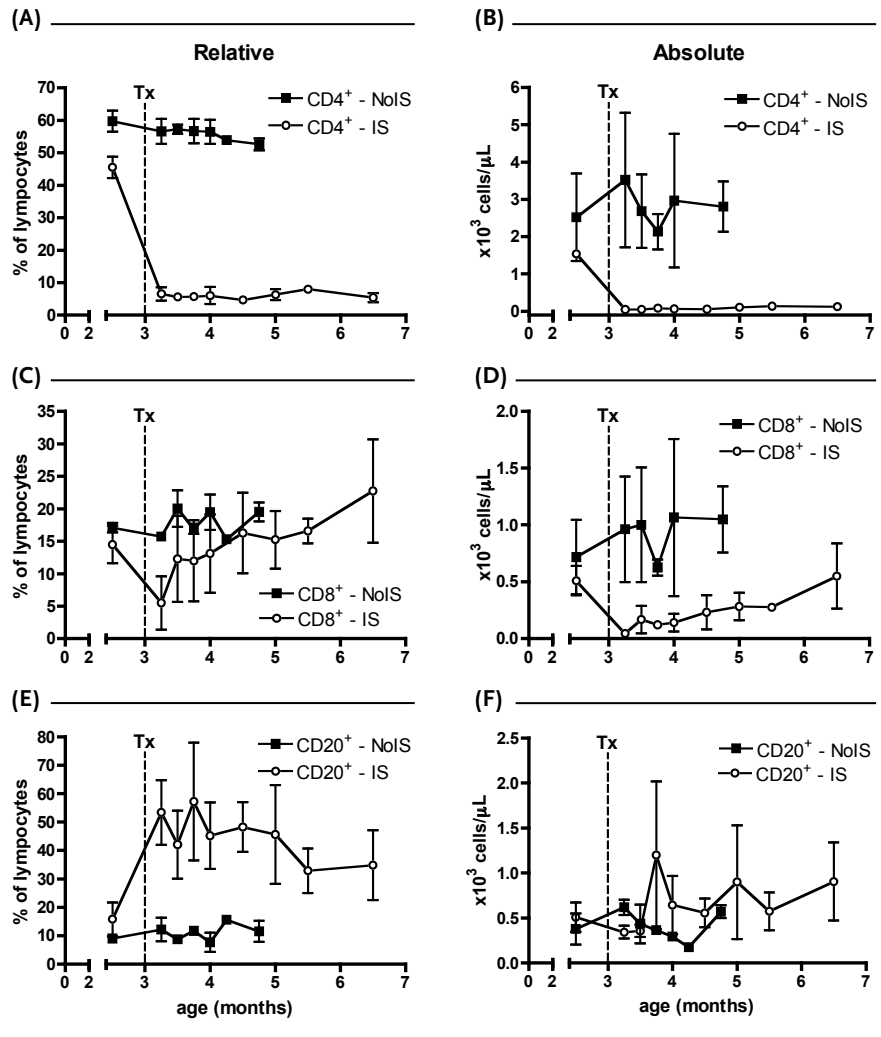
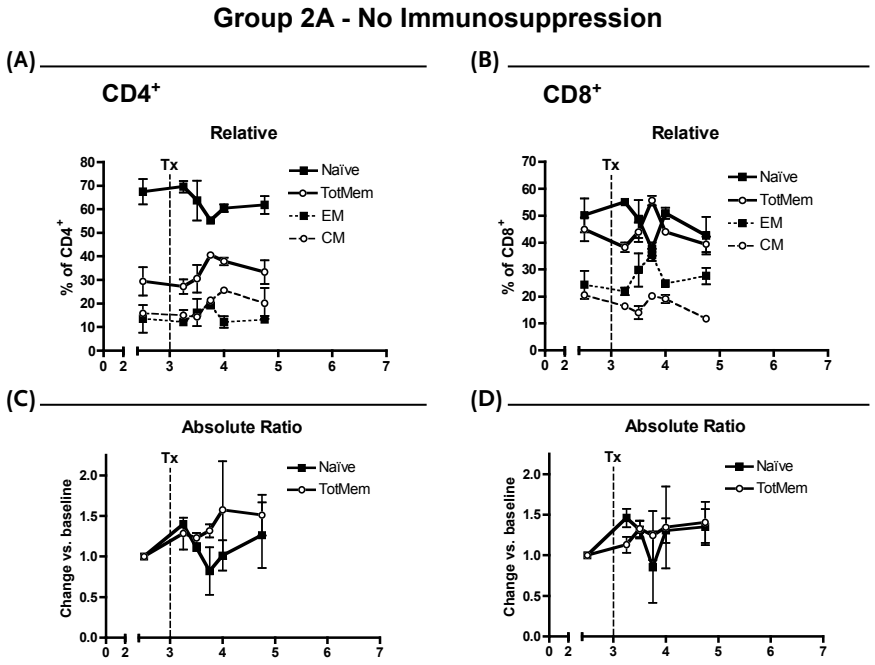


FIGURE 5 | CHANGES IN CD4+ AND CD8+ T CELLS, AND CD20+ B CELLS IN INFANT BABOONS AFTER TX WITH AND WITHOUT IMMUNOSUPPRESSION (GROUP 2): Left panels show percentages of total $CD4^+$ T cells (A), $CD8^+$ T cells (C), and B cells (E), right panels show corresponding absolute numbers (B, D, F). Data are presented in the absence (No IS – Group 2A) or presence (IS – Group 2B) of immunosuppressive therapy.



Group 2B: Tx in presence of IS

Induction therapy with ATG resulted in depletion of >95% of $CD4^+$ cells, and >90% of $CD8^+$ cells in 3 infant baboons (Figures 5B, 5D). $CD4^+$ cells were maintained at low levels with anti-CD154 mAb and MMF, as has been previously described in older animals (19) (Figures 5A, 5B). $CD8^+$ cells recovered during the 3.5 months follow-up to levels comparable with those measured at baseline (Figures 5C, 5D). The increased proportion of B cells within the lymphocyte gate (Figure 5E) was a consequence of depletion of T cells, as no significant change in absolute numbers of B cells was noted (Figure 5F).

During follow-up, pronounced changes in relative numbers of T_N, T_{TotMem}, T_{EM}, and T_{CM} were seen. $CD4^+$ T_N fell from 55.8±6.6% at baseline to 14.8±4.9% at 6 weeks post-Tx (repeated measures ANOVA, p=0.013, Figure 6E). During this period, $CD4^+$ T_{TotMem} had increased from 42.0±6.6% to 82.2±6.7% (repeated measures ANOVA, p=0.044). At the age of 4 months, a major shift from T_N to T_{TotMem} as part of the natural development of the immune system would no longer be expected (compare with Figure 2B), and is therefore likely to be caused by lymphocyte depletion and repopulation phenomena. Although absolute numbers of $CD4^+$ cells were still low, the altered proportions of T_N and T_{TotMem} resulted in the earlier repopulation of T_{TotMem} than T_N (Figure 6G). Starting 2 months after Tx (at age 5 months), the relative numbers largely reversed to those measured pre-Tx (Figure 6E).

The changes in $CD8^+$ T_N and T_{TotMem} followed similar patterns to those in $CD4^+$ cells. $CD8^+$ T_N levels fell from 38.9±6.3% at baseline to 21.1±3.6% at 3 weeks post-Tx (repeated measures ANOVA, p=0.003, Figure 6F). $CD8^+$ T_{TotMem} transiently increased from 58.2±5.8% to 71.3±3.2% (repeated measures ANOVA, p=0.027), which, however, did not greatly influence absolute numbers (Figure 6H). Six weeks post-Tx reversal to proportions comparable with baseline had occurred.

When changes in T_{Reg} after Tx without (Group 2A) and with (Group 2B) IS were compared, opposite phenomena were observed. While without IS the percentage of T_{Reg} among $CD4^+$ cells did not change (Figure 7A), after T cell depletion and chronic IS a relative increase in T_{Reg} among $CD4^+$ cells was seen (although non-significant, Figure 7D). However, this led neither to an increase in percentage among $CD4^+CD25^{hi}$ cells, nor to an increase in absolute numbers

FIGURE 6 (ON LEFT PAGE) | NAÏVE AND MEMORY SUBPOPULATIONS AMONG $CD4^+$ AND $CD8^+$ T CELLS IN INFANT BABOONS AFTER TX WITH AND WITHOUT IMMUNOSUPPRESSION (GROUP 2): (A-D) Group 2A – No Immunosuppression. (E- H) Group 2B – Immunosuppression. Shifts toward a memory phenotype (E, F) and significantly greater expansion of memory T cells (G) indicated homeostatic proliferation after induction therapy with anti-thymocyte globulin. *p<0.05 vs. Naïve. (TotMem = total memory T cells; CM = central memory T cells; EM = effector memory T cells).

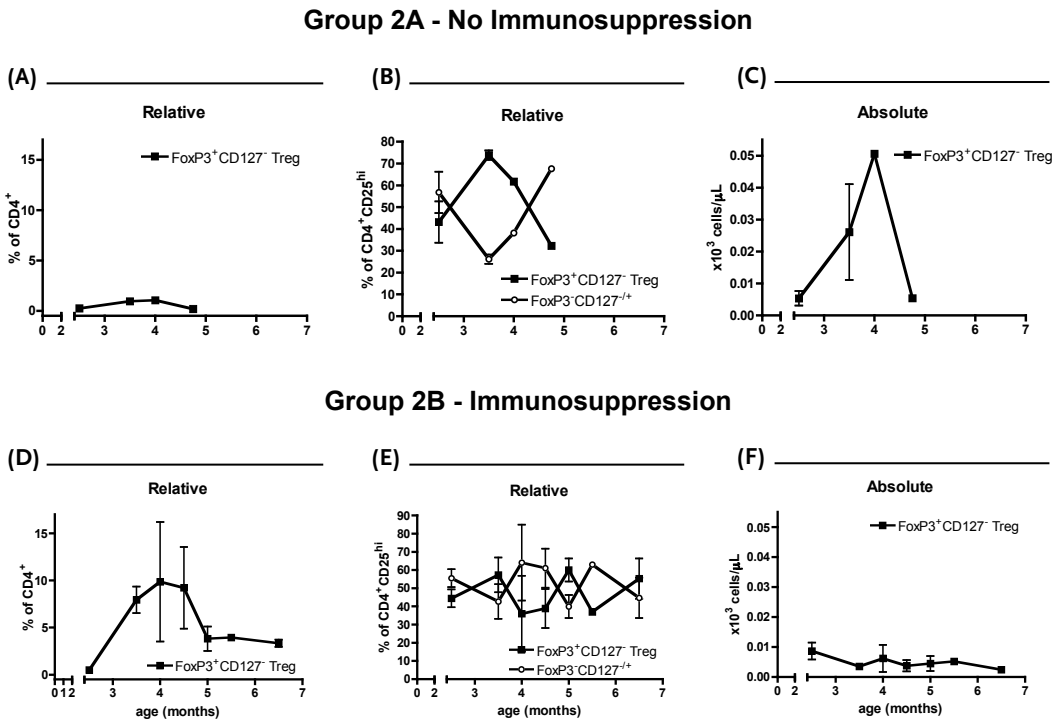


FIGURE 7 | REGULATORY T CELLS IN INFANT BABOONS AFTER TX (GROUP 2): Proportions among $CD4^+$ (A, D) and $CD4^+CD25^{hi}$ (B, D) cells, and absolute numbers (C, F) in the absence (Group 2A - No Immunosuppression) or presence (Group 2B - Immunosuppression) of immunosuppressive therapy. (Treg = regulatory T cells).

($p>0.05$, Figures 7E, 7F). This indicated that FoxP3 expression did not increase among $CD4^+CD25^{hi}$ cells, and that the increase among $CD4^+$ cells was more likely a result of lymphocyte depletion, which relatively spared T_{Reg}.

The applied immunosuppressive protocol (ATG, anti-CD154 mAb, MMF) adequately suppressed the response in the MLR with donor-type stimulation during the course of follow-up (Figure 4).

Discussion

The aims of the studies here presented were to verify if infant NHPs (i) undergo LIP to expand their T cell numbers after birth, and (ii) are prone to the induction of immunologic tolerance to transplant antigens. Both phenomena have been previously documented in laboratory rodents, but it remained unknown if these fundamental immunologic principles are similar in NHPs and humans. We observed that infant baboons were relatively lymphopenic at birth. The initial naïve immune status and subsequent LIP or 'homeostatic proliferation' of T cells, which generated memory T cells, did not affect the functionality of the immune system when challenged with AB-I baboon or WT pig antigens. IS including lymphocyte depletion was effective in the suppression of cellular rejection, but caused significant shifts in T cell phenotype.

During the first 4 months of life of baboons, the blood T lymphocyte pool expanded three to seven fold. Numbers of $CD8^+$ cells increased more substantially than the $CD4^+$ subpopulation (which relative numbers actually slightly declined, as previously reported in humans [5, 14]). Although early in life the thymus still contributes importantly to T cell homeostasis, in our studies evidenced by increasing T_N, a relatively greater expansion of memory T lymphocytes was seen. Two plausible mechanisms could have contributed to this observation, being (i) LIP, and (ii) exposure to antigens in the environment, likely as a result of intestinal colonization (20), while further antigen exposure was limited in the specific pathogen-free housing environment. The observation that the shift toward increased T_{TotMem} was transient and reversed after approximately 6 months of age suggests a dominant role for LIP.

Heart Tx is the only life-saving therapy in certain congenital cardiac abnormalities. In neonates, a heart can be successfully transplanted across the ABO-blood group barrier (6-9), likely because the natural antibodies against non-self blood groups do not develop until later. Moreover, after Tx the development of antibodies against the donor blood group never occurred, while antibodies against non-donor, non-self readily appeared. This indicates that B cell tolerance was induced (11), although it should be noted that recipients were thymectomized and remained fully immunosuppressed during follow-up. It is unknown if the T cell compartment contributed to this tolerant state,

and whether it would be possible to reduce the (T cell-directed) IS in infant Tx recipients. We therefore established the infant baboon artery patch Tx model to undertake immunologic studies.

Because heart Tx in infants is limited by the scarcity of size-matched donor hearts, we also investigated xenoTx, i.e., pig-to-human, which could provide at least a 'bridge' for the patient until a suitable human donor heart becomes available (21). The observation that natural anti-pig antibodies develop in a similar pattern as anti-A or B blood group antibodies led us to hypothesize there might be an opportunity for tolerance induction to pig antigens (22). The outcomes regarding B cell tolerance will be separately reported (Dons EM et al., manuscript submitted).

Regarding T cell immunity, no major phenotypic changes in the T_N and T_{TotMem} compartments occurred after Tx without IS during six weeks of follow-up. Donor-type specific sensitization was induced, as proven by an increased response in the MLR, the appearance of donor-type specific antibodies in serum, and pathologic changes in the graft. These results indicate that the T cell immune system of baboon infants, although still antigen-naïve, can readily mount an immune response against donor antigens.

The applied IS prevented the induction of anti-donor-type antibodies, and reduced the response in the MLR against donor-type stimulators, as well as against third-party stimulators. Signs of selective tolerance induction were therefore not observed, as this is traditionally defined as specific unresponsiveness to a specific foreign (Tx) antigen while maintaining reactivity to other (third-party) antigens (23). Lymphocyte depletion with ATG caused significant shifts in naïve and memory T cells. Thereafter, peripheral blood was mainly repopulated with memory T lymphocytes, a phenomenon known as homeostatic repopulation (24, 25). These vigorous phenotypic changes seem to have been overruled by the immunosuppressive regimen with respect to their *in vitro* functional potential in the MLR. However, it should be acknowledged that at the time of the major T_N - T_{TotMem} phenotypic shifts, T cell numbers were too low to harvest sufficient numbers to perform MLR. The intervals between MLR results are thus larger than the time required for phenotypic changes. We also acknowledge that the number of animals in our studies is small and that further conclusions might not be justified.

Homeostatic repopulation of memory T cells after lymphocyte depletion has been observed in patients with organ transplants (26, 27), and can result in acute cellular rejection with T_{TotMem} predominating in peripheral blood and in graft histology (27). Memory cells can be cross-reactive with graft antigens and cause rejection with reduced need for antigen presentation and costimulation,

so called 'heterologous immunity' (23, 28). This represents a clinically-relevant problem, e.g., when weaning of immunosuppression is attempted. Moreover, memory cells have been found to represent a barrier to Tx tolerance (23, 29).

The ideal panel of conjugated mAbs used to identify lymphocyte subsets by their cell surface antigens remains a topic of debate. In our studies, we opted for differentiation of T_N and T_{TotMem} by mAbs against CD45RA and CD62L, a strategy that is well-established in humans (28, 30) and NHP (31-34), and that we previously successfully applied in cynomolgus monkeys (35). However, Pitcher et al. postulated that memory T cells in rhesus monkeys can best be determined by anti-CD95 and CD28 staining (16). The different strategies, however, stained very significantly overlapping cell populations (16, 36), leading us to conclude that CD45RA and CD62L can be reliably used in baboon studies.

In conclusion, after birth the immune system of infant baboons is relatively lymphopenic and naïve, and the numbers of T lymphocytes significantly increase with a transient appearance of memory T cells. We hereby confirmed that neonatal NHPs undergo LIP to establish T lymphocyte homeostasis. After artery patch Tx in baboons 3 months of age, an age at which natural Abs against ABO-blood group and pig antigens are still practically undetectable (22), the T cell immune system was able to readily and actively mount an immune response against donor-type antigens. On the basis of the assays performed, we therefore did not identify a T cell 'window of opportunity' for tolerance induction. However, the PBMC MLR is a crude functional assay, and more subtle signs of immune tolerance, or opportunities to achieve so, may have been missed. More detailed investigations in this regard are warranted. IS including lymphocyte depletion was associated with the expansion of memory T cells that may actually form an obstacle if immunologic tolerance is the goal.

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DISCUSSION

CHAPTER 9

General discussion

ABSTRACT

The major aims of the research reported in this dissertation were to investigate the conditions that enable the successful Tx of pig islets into diabetic NHPs, with the ultimate goal to cure patients with T1D. This thesis describes the available knowledge about the early inflammatory response that hampers islet engraftment. Its mechanisms were further investigated in a simplified *in vitro* model. Diabetic monkeys were transplanted with islets from WT pigs and islets from pigs transgenic for hCD46, a human CRP. Alternative anatomical sites for the portal vein as site of islet injection were reviewed, and the GSMS as the recipient site for islet Tx was explored in pigs. The immunologic effects of alemtuzumab for induction therapy were for the first time investigated in NHPs. Finally, the immune system of infant NHPs was studied to verify two fundamental immunologic principles with consequences for Tx, previously observed in laboratory mice, being neonatal lymphopenia-induced proliferation and opportunities for tolerance induction. In this general discussion the main findings are interpreted, and implications for future research are discussed.

Main Results & Interpretation

MECHANISMS OF EARLY GRAFT LOSS AFTER ISLET TRANSPLANTATION INTO THE PORTAL VEIN

A major limitation of the success of islet Tx into the portal vein is the early and rapid loss of up to 70% of the graft (1). This can result in what has previously been described as 'early graft loss' or 'primary non-function'. Its immediate occurrence suggests a role for inflammatory contributors, such as coagulation, platelet activation and complement activation, as well as innate immunological factors, such as neutrophilic granulocyte and monocyte infiltration (**Chapter 2**). The interplay between these events is complex and has been termed the 'instant blood-mediated inflammatory reaction' (IBMIR) (2). First, it was hypothesized that coagulation was the driving force in IBMIR, because (i) isolated islets were found to express tissue factor (3), (ii) inhibition of thrombin prevented islet damage *in vitro* to a large extent (4), and (iii) islets exposed to serum (i.e., in absence of coagulation factors) suffered reduced damage (5). However, anticoagulation with heparin (3) or with more experimental anticoagulants, such as LMW-DS, did not prevent early islet loss *in vivo* (6). Also in subsequent *in vitro* studies it was concluded that anticoagulation alone is insufficient to inhibit IBMIR and early islet loss (7, 8). When human and pig islets were exposed to fresh whole blood in conditions of autoTx, alloTx and xenoTx (**Chapter 3**), clotting occurred equally rapidly in all tested conditions. Anticoagulation did not prevent cell membrane damage and leakage of C-peptide. Nevertheless, anticoagulation to prevent portal vein thrombosis during intraportal islet infusion remains an obvious necessity.

Furthermore, a central role for complement activation in IBMIR has been postulated (2, 5, 9-11). In what was considered the most advanced *in vitro* model - a tubing loop model in which a blood flow could be created - complement activation by, and deposition on human and pig islets was seen in the absence of binding of IgM and IgG antibodies (2, 9). Although a role for antibodies could not be excluded (5, 10), it was generally believed that complement activation after islet Tx was not initiated by antibody binding. It was therefore concluded that hyperacute rejection, in which natural preformed antibodies bind to the graft and activate complement through the classical pathway, resulting in cell lysis, is not a contributory mechanism in IBMIR. It was more likely that

complement activation in IBMIR occurred through the alternative pathway, making it a non-specific phenomenon driven by coagulation, platelet activation and inflammation (**Chapter 2**).

Concerning coagulation and platelet consumption, the studies in this thesis confirmed their non-specific character, as they occurred equally in autoTx, alloTx and xenoTx. However, in a direct comparison, C-peptide release was significantly higher when pig islets were exposed to human blood, when compared with human islets exposed to human blood, indicating more damage to pig islet cells than to human cells (**Chapter 3**). In search for the cause of this difference, we found that IgM and IgG antibodies had bound to the islet surface. In addition, plasma (i.e., serum including coagulation factors, antibodies and complement) was sufficient to cause lysis of pig cells. Because the expression of the Gal epitope - the pig epitope against which natural antibodies in humans are mostly directed (12) - is low on adult pig islets (13), it is likely that antibody binding to non-Gal antigens caused complement activation. The resulting cell lysis was effectively reduced when IgM was neutralized, when heat-inactivated plasma (i.e., serum including coagulation factors and antibodies, but in absence of complement) was used, and when complement activation was inhibited by compstatin.

Using a large particle flow cytometry technique, the group that initially reported no role for preformed antibodies recently reported deposition of IgM and IgG antibodies on human and pig islets in *in vitro* models of IBMIR (14, 15). Although in our model the binding of antibodies to human islets was minimal in comparison with pig islets, a role for preformed antibodies in clinical islet alloTx of human islets has been suggested. In patients with preformed antibodies, e.g., when there are high antibody titers against foreign HLA, success rates to reach normoglycemia were lower (16-18).

Altogether, these results lead to the conclusion that IBMIR is less non-specific than previously anticipated. It is specifically more destructive to pig islets and humoral immunity plays an important role above and beyond pro-coagulative events, in a fashion comparable to hyperacute rejection. Humoral immunity needs to be efficiently downregulated if islet alloTx and, particularly, xenoTx of pig islets into the portal vein is to become a successful form of therapy for T1D.

hCD46 EXPRESSION ON PIG ISLETS EXTENDS THEIR SURVIVAL AFTER XENOTRANSPLANTATION

In the investigations leading to this dissertation, for the first time the survival of a functional islet xenograft longer than 1 year was achieved (at which time the experiment was electively terminated) in a chemically-induced diabetic cynomolgus monkey (**Chapter 4**). In another 3 monkeys, normoglycemia was achieved for more than 3 months. They were neither limited in diet nor received

exogenous insulin injections for the full time of follow-up. The adult pig islets, transgenic for the expression of hCD46, that these monkeys received clearly demonstrated an advantage over WT pig islets when transplanted intraportally into diabetic, immunosuppressed NHPs.

Recipient monkeys were prepared for Tx with an anticoagulant, anti-inflammatory treatment protocol, developed based on our group's previous experiences with IBMIR (6). In those experiments, IBMIR could not be successfully avoided, but an increase in the number of transplanted islets to 80,000 IEQ/kg body weight allowed sufficient islets to survive the IBMIR and establish normoglycemia (6). In the present experiments, the hypothesis was tested that the use of pig islets expressing hCD46 in combination with the anti-inflammatory protocol, would reduce the effects of complement activation during the IBMIR, thus more efficiently restoring normoglycemia in monkeys. Nonetheless, in both groups (transplanted with WT or hCD46 islets), we observed acute early islet loss characterized by an equally high peak in serum C-peptide levels and hypoglycemia, indicating cell lysis and leakage of stored insulin. Also in the *in vitro* experiments (**Chapter 3**), hCD46 expression was unable to prevent leakage of insulin and C-peptide from pig islet cells. The hypothesis that hCD46 would help to increase the number of infused islets to survive the IBMIR was therefore not confirmed. It appeared that, although high numbers of porcine islets turned out to be necessary, only a marginal mass of surviving cells is sufficient to sustain normoglycemia.

Nevertheless, once the immediate post-Tx phase had passed, engrafted hCD46 porcine islets functioned significantly longer than their WT equivalents, the latter only able to maintain normoglycemia for a maximum of 36 days. In both groups, exactly the same protocol for immunosuppressive therapy was used, consisting of induction with ATG, and maintenance with an anti-CD154 mAb and MMF. It was previously demonstrated that this regimen efficaciously suppressed the NHP T lymphocyte response against pig xenoantigens (19). In the absence of T cell-directed immunosuppression, acute cellular rejection is the dominant nature of islet xenograft rejection (20). After necropsy of our transplanted monkeys, regardless of the islet source, time of necropsy and the functional status of the graft, no cellular infiltration was observed in or around islets on histologic analysis of the liver, confirming the efficacy of the T cell-immunosuppressive therapy.

Binding of IgG, however, was observed on WT islets as well as on islets expressing hCD46. However, C4d immunostaining for complement activation was present only on WT islets, suggesting that hCD46 may have exerted a protective role against humoral rejection by low levels of natural or induced anti-pig antibodies. These antibodies were present in monkey sera in low levels before and after Tx and, although they did not increase after Tx, may have been

the cause for loss of WT islet function due to humoral rejection. Comparably, a case of humoral rejection after islet alloTx in a human patient has recently been described (21).

THE ANATOMICAL SITE FOR ISLET TRANSPLANTATION

Because the IBMIR occurs when islets are in direct contact with the blood stream, an alternative non-intravascular site for islet Tx has been the subject of many investigations, as reviewed in **Chapter 5**. The actual islet cell mass to be transplanted is usually smaller than 2mL in volume, and therefore any site that can accommodate this small volume can theoretically be a candidate to harbor the graft. We hypothesized several favorable conditions for islet survival in the GSMS, such as an extravascular, but well-vascularized microenvironment for oxygen and nutrient supply, and the avoidance of IBMIR. In studies of islet alloTx in diabetic pigs (**Chapter 6**) the technique for islet Tx into the GSMS through an endoscope was successfully established. Endoscopic Tx could be performed without complications - it especially avoided the risk of bleeding and thrombosis related to intraportal Tx - and took only 5-10 minutes in the hands of an experienced endoscopist. Monitoring demonstrated that the peak release of C-peptide within the first hour after Tx was significantly lower than after Tx into the portal vein. From this observation we concluded that the loss of islets from IBMIR was prevented. After 4 weeks, blood glucose values were significantly lower while less exogenous insulin was administered. At necropsy, viable islets were found in the GSMS. In our laboratory, we have meanwhile demonstrated that biopsies of islets in the GSMS of pigs can be taken safely, again with a minimally-invasive endoscopic approach (Drs. Hidetaka Hara and Minoru Fujita, University of Pittsburgh, unpublished data). A simple and safe procedure for Tx and biopsy would allow for careful monitoring for rejection, and if indicated, re-Tx could be carried out with minimal risks.

Although the results in pigs have been encouraging, unanswered questions need to be addressed before this technique can be applied clinically. Applying immunosuppression in pigs proved difficult because of their susceptibility to severe infections. When immunosuppression was reduced or discontinued, cellular infiltration around the islets occurred. Immune responses and the nature of graft rejection in the GSMS, such as the role of mucosa-associated lymphoid tissue (MALT), require investigation. Several pig recipients suffered from severe hypoglycemic episodes and it is unclear if this was caused by infection, Tx of excessively high numbers of islets, or yet unknown metabolic consequences of islet Tx into the GSMS. In future experiments, these issues will need to be clarified, preferably in experiments in NHPs. In addition, as the loss of islets from IBMIR in the GSMS is likely to be avoided, it will be important to assess if insulin-independence can be successfully achieved with the Tx of fewer islets than needed for Tx into the portal vein. This would enable

fewer pancreases to be used for a greater number of patients. If the GSMS is confirmed to be a preferable site, patients with T1D could immediately receive islet alloTx using this approach.

Recently, interesting developments regarding other sites for islet Tx have been reported. In diabetic monkeys, functional alloTx of islets loaded onto a biodegradable scaffold that was wrapped with omentum and placed between the abdominal muscles, was achieved (22). The promising results in a NHP model indicate clinical potential for this approach. The Tx of islets into the brachioradialis muscle has already reached the clinical stage in patients that underwent pancreatectomy and were subsequently autotransplanted with their isolated islets (23, 24). A new approach not mentioned in **Chapter 5** has recently been reported. In mice, islet injection into bone marrow cured diabetes for longer than a year (25). While this approach needs to be confirmed in large animals, the intra-bone marrow injection procedure has already been proved feasible in humans for injecting umbilical cord blood cells (26).

Eventually, the liver as the site for islet Tx will be replaced by a site where the balance between delivery of oxygen and nutrients to the islets, and shielding of the islets from an immune attack is most favorable.

IMMUNOSUPPRESSIVE PROTOCOLS FOR ISLET XENOTRANSPLANTATION

Hitherto, intense immunosuppressive strategies have been required to control rejection of a xenograft, whether being an islet or an organ graft (19, 27-29). Adverse events associated with immunosuppression prematurely terminated experiments of islet xenoTx (27, 28). In the experiments in **Chapter 4**, the (chronic) use of corticosteroids or calcineurin inhibitors was avoided, as they have detrimental effects on transplanted islets (30). The applied regimen of ATG, anti-CD154 mAb and MMF was relatively mild, yet efficacious to block T cell rejection, and was possibly 'assisted' by the additional effect of hCD46 on blocking humoral rejection. Consequently, the applied immunosuppression did not lead to drug-related morbidity and mortality in monkey recipients, thereby advancing the field of islet xenoTx toward clinical application. In fact, monkeys remained healthy and gained weight according to the standards for their natural development.

However, an anti-CD154 mAb for costimulatory blockade was used. In view of its potential thrombogenic effects (31-34), this mAb is unlikely to be approved for clinical use, which illustrates the necessity to search for safe yet efficacious immunosuppression for xenoTx. At the University of Pittsburgh, excellent results have been obtained with the use of alemtuzumab for induction therapy in clinical alloTx (35, 36), which may allow for a low level of maintenance immunosuppression (36). With an induction agent as powerful

as alemtuzumab, a weaker, but clinically-acceptable, agent for costimulation blockade (such as CTLA4-Ig) might suffice. The preclinical use of alemtuzumab is, however, complicated by the observation that many NHP species express CD52 on RBC in addition to the targeted lymphocytes (37), risking life-threatening hemolysis if this agent is used in these animals.

Using an agglutination screening assay, only cynomolgus monkeys from the island of Java, Indonesia, appeared negative for CD52 expression on RBC, whereafter a dose-efficacy study was undertaken (**Chapter 7**). Because the affinity of alemtuzumab for monkey CD52 was much lower than for human CD52, monkeys were administered a dose 20x higher than the clinical dose. This resulted in excellent lymphocyte depletion, which was greater than after administration of ATG. In addition, B cells were completely depleted from the blood, a result not achieved by ATG. Recovery of T cells was faster than the recovery of lymphocytes in patients, but could be suppressed with repetitive injections of a reduced dose and the addition of chronic MMF therapy. Despite the slightly different responses to alemtuzumab in monkeys and humans, the establishment of a NHP model for alemtuzumab is important. It allows us to explore its effects on the immune system, to address the question of whether it facilitates the reduction of maintenance immunosuppressive therapy and/or the development of transplant tolerance, and to investigate if alemtuzumab could be the cornerstone of a patient-friendly regimen to ensure xenograft survival. Of importance, once these questions have been answered, application in human patients will be readily possible, as the use of alemtuzumab in humans is clinically approved and well-established. The experience with alemtuzumab and islet alloTx is increasing (38, 39), and initial results indicate an extension in the length of insulin-independence, which is currently longer than 3 years in 70% of transplanted patients (Dr. Camillo Ricordi, University of Miami, personal communication).

MEMORY T LYMPHOCYTES AND (XENO)TRANSPLANTATION

Although after Tx the transplant antigens are exposed to the recipient's immune system for the first time, the immune response against transplant antigens has been shown not to be truly primary (40). Antigen-experienced memory T cells can cross-react with graft antigens and cause rejection with reduced need for antigen presentation and costimulation, so called 'heterologous immunity' (41, 42). The expansion of memory T cells after lymphocyte depletion (homeostatic proliferation) has been observed in patients with organ transplants who have received alemtuzumab as induction therapy (43, 44), in some cases resulting in acute cellular rejection (44). Although the clinical short-term results with alemtuzumab have been excellent, the changes it causes to the naïve and memory T lymphocyte pools may prove problematic if weaning of maintenance immunosuppression is attempted. Indeed, in a study in which chronic

immunosuppression was reduced, the incidence of acute cellular rejection rose to 25% of patients (36). In addition, memory T cells have proven to be a barrier to immunologic transplant tolerance, the ultimate goal of Tx research (45).

In the studies of alemtuzumab in monkeys (**Chapter 7**) and ATG in infant baboons (**Chapter 8**), homeostatic proliferation after induced lymphopenia was observed. In addition, it occurred during the first 4 months of life of healthy, untreated infant baboons, in which it likely resolves their relatively lymphopenic condition at birth. Although being naïve at birth, the infant baboon immune system was able to adequately respond to transplanted antigens. The induction of a T cell-tolerant state (as can be achieved for B cells after heart Tx in neonates [46]) is therefore unlikely to be achieved after alloTx or xenoTx.

Methodological Considerations

The labor intensity of Tx experiments in large animals (e.g., NHPs and pigs), and therefore its cost, is immense. The number of experiments in preclinical studies, such as presented in this dissertation, is therefore limited and aimed mainly at establishing efficacious, safe and reproducible methodology for innovative new treatment options. Providing statistical evidence that one treatment is favorable over another, the foundation of evidence-based medicine, is not always feasible. Nevertheless, large animal models, especially those using NHPs, are indispensable for the development of new treatment strategies and are often considered the ultimate evidence that those strategies have potential for clinical application.

Clinical Applications of Islet Xenotransplantation

During the last decade, a number of clinical trials of islet xenoTx have been reported. In Mexico, pediatric diabetic patients were transplanted with pig islets that were shielded from the recipient's immune system by transplanting them in a stainless steel chamber placed under the skin (47). In Russia and New Zealand, pig islets were individually encapsulated in alginate and transplanted into the peritoneal cavity (48, 49). These immuno-isolating approaches avoided the necessity for immunosuppressive therapy. However, regardless of the results of such trials, lively discussions about their justification on regulatory and ethical grounds have emerged in the xenoTx community (49-51). Where the level of regulatory oversight on the Mexican and Russian trials has been unclear, the trial in New Zealand is being carefully regulated by legislation of its Ministry of Health. Nevertheless, none of these clinical studies have been preceded by peer-reviewed preclinical data in NHPs proving the efficacy of the therapy. The International Xenotransplantation Association and the World Health Organization have both stressed that, in addition to the need for strict procedures to guarantee a safe pig product, patients should only be exposed to islet xenoTx if there is a relatively high expectation of benefit from it (52-54). Sound pre-clinical data from experiments in NHPs remain the best available proof of potential therapeutic benefit.

Future Directions

Ten-year insulin-independent survival after islet alloTx has been achieved (55). Nevertheless, the quantities of available human donor islets are inadequate to treat more than a small percentage of patients with T1D. Islets from pigs can solve this shortage and can function adequately in NHPs, as supported by previous reports and by encouraging data in this dissertation.

In future experiments, we will focus on developing a protocol for islet xenoTx in the optimal anatomical site with a safe and efficacious immunosuppressive protocol. In particular, we are planning to investigate the GSMS as the site for islet Tx in NHPs, and the use of an alemtuzumab-based immunosuppression regimen. Pigs with various genetic modifications in addition to the absence of the Gal epitope and the expression of hCD46 are now available to us (56). Islets from those genetically-engineered pigs express genes that help to control coagulation, inflammation and rejection, and may help to reduce both the IBMIR and the need for exogenous immunosuppression. We will investigate islet-like cell clusters from neonatal pigs (7-14 days after birth) that are rather easier to isolate than adult pig islets and can proliferate after Tx. If clinical pig islet xenoTx were to be carried out on a large scale, the use of neonatal pigs would greatly reduce the expense, as they do not need to be housed until adulthood.

International regulations for clinical trials are being established (57), and the first clinical trials are underway. It is expected that more clinical studies will be started in the next few years. Islet xenoTx has the potential to become the treatment of choice for T1D.

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SUM

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CHAPTER 10

Summary / Samenvatting

Summary

This dissertation describes studies on the conditions required for successful xenotransplantation of islets of Langerhans in order to provide patients a cure for type 1 diabetes. In **Chapter 1**, the current status of clinical islet allotransplantation, and of preclinical islet xenotransplantation, are introduced. Several events hamper the success of islet allotransplantation, such as the provocation of an 'instant blood-mediated inflammatory reaction' (IBMIR) after islet injection into the portal vein, loss of islet function over time, and insufficient numbers of available human donor organs to treat more than a small percentage of patients with type 1 diabetes. Xenotransplantation of pig islets has successfully cured diabetes in non-human primates, but unacceptably intense immunosuppressive therapy needs to be applied to prevent rejection. Therefore the aims of this research were to increase our understanding of the IBMIR, to prevent the IBMIR and humoral rejection by transplanting islets from pigs that are transgenic for a human complement-regulatory protein, to explore a new anatomical site for islet transplantation, and to work on an immunosuppressive regimen that will prevent xenograft rejection yet can safely be applied in patients.

Chapter 2 describes the results of a literature search on factors that contribute to early islet damage in the IBMIR, and discusses strategies to prevent this damage. The initial loss of a large number of islets after transplantation into the portal vein is caused by an inflammatory reaction composed of activation of coagulation and complement, platelet adhesion, and involvement of neutrophils and macrophages. It occurs after allotransplanation and is equally, if not more, of a problem after xenotransplantation. Minimization of this reaction would seems essential, and may result in successful clinical transplantation with a lower islet mass, thereby contributing to better utilization of the available human donor pancreases. Even in xenotransplantation, where the number of islets is theoretically limitless, IBMIR should be minimized if islet transplantation is to become a practical therapeutic option for type 1 diabetes.

In **Chapter 3**, an *in vitro* model was established to further investigate the IBMIR. It was found that isolated islets non-specifically induce rapid clotting after direct contact with fresh blood in conditions of autotransplantation, allotransplantation, and xenotransplantation. However, islet cell damage was more

severe as islet and blood compatibility diminished, with substantial islet injury after exposure of pig islets to human blood. Antibody binding and complement activation were more significant after exposure of pig islets to blood, than when the same number of human islets were exposed to blood. Islet damage occurred even after exposure to plasma only. Specific complement inactivation and neutralization of IgM substantially prevented islet damage. These observations indicate the importance of humoral immunity in xenogeneic IBMIR.

Chapter 4 describes the experiments of islet xenotransplantation in diabetic non-human primates. The effect of the transgenic expression of human CD46 (hCD46), a complement-regulatory protein, on pig islets was investigated. Monkeys receiving wild-type islets served as controls. The transplantation of islets from hCD46 transgenic pigs resulted in graft survival and insulin-independent normoglycemia in four of five monkeys for the 3 months follow-up of the experiment. One randomly selected recipient was followed for >12 months and showed excellent graft function until the experiment was electively terminated. These results were significantly better than after transplantation of wild-type islets. Although inhibition of complement activation by hCD46 did not substantially reduce the initial loss of islet mass, hCD46 seemed to prevent humoral rejection, likely induced by low levels of anti-pig antibodies. It thereby reduced the need for immunosuppression to preserve a sufficient islet mass for the long-term maintenance of normoglycemia.

Because the volume of the islet graft mass is only small and transplantation into the portal vein causes IBMIR, many other anatomical sites for islet transplantation have been subject of investigation, as reviewed in **Chapter 5**. In **Chapter 6**, the gastric submucosal space as a site for islet transplantation was explored in experiments using diabetic pigs. It appeared feasible to inject the islet graft into the gastric submucosal space using an endoscope. The procedure was quick, minimally-invasive, and free from complications. IBMIR was avoided, in contrast with observations in control pigs that received islets in the portal vein.

The aim of **Chapter 7** was to establish the use of alemtuzumab, anti-CD52 monoclonal antibodies, in non-human primates. Although in patients highly efficacious as a lymphocyte-depleting agent, in non-human primates its use might be complicated by hemolysis, as the target CD52 molecule is often expressed on red blood cells too. With an agglutination screening assay, cynomolgus monkeys negative for CD52 on red blood cells were identified and high doses of alemtuzumab could be administered safely. Near-complete depletion of T and B lymphocytes from blood and lymph nodes was achieved and was more profound than in monkeys treated with anti-thymocyte globulin. Repopulation was suppressed by weekly injections and with mycophenolate mofetil. Among repopulating cells, a phenotypic shift towards memory T cells was observed.

This preclinical model offers opportunities to investigate lymphocyte depletion/repopulation phenomena, as well as the feasibility of alemtuzumab to become the cornerstone of a safe protocol for xenograft rejection.

In **Chapter 8**, the immune system of infant baboons was studied. The aim was to verify two fundamental immunologic principles previously observed in mice, being neonatal lymphopenia-induced proliferation of memory T cells, and the ability to immunologically tolerate transplant antigens. Using flowcytometry, it was found that baboons are born relatively lymphopenic and subsequently expand their initially naïve T cell pool with the proliferation of memory T cells. After transplantation of an artery patch allograft or xenograft, non-immunosuppressed recipients readily and actively mounted an immune response against donor-type antigens. Indications that T cell immunological tolerance could be achieved were not observed. Immunosuppression aiming at lymphocyte depletion and cellular rejection prevented T cell-mediated rejection. After lymphocyte depletion, the homeostatic proliferation of memory T cells was observed, which may actually form an obstacle if tapering of the immunosuppressive therapy, or ultimately, immunologic tolerance are to be achieved.

Chapter 9 provides an overall scientific interpretation of the studies reported in this dissertation. In addition, methodological considerations and future directions for preclinical and clinical studies are discussed.

Samenvatting

Dit proefschrift beschrijft studies gericht op het onderzoeken van de voorwaarden voor een veilige en effectieve toepassing van xenotransplantatie van eilandjes van Langerhans ter genezing van diabetes type 1. In **Hoofdstuk 1** wordt de huidige status van klinische allotransplantatie en experimentele xenotransplantatie geïntroduceerd. Het succes van allotransplantatie van menselijke eilandjes is beperkt door (i) het optreden van een ontstekingsreactie na injectie in de bloedstroom van de poortader die eilandjes naar de lever meevoert, (ii) geleidelijk functieverlies met de tijd en (iii) het tekort aan menselijke orgaandonoren. Xenotransplantatie van varkenseilandjes kan diabetes in apen genezen, maar vereiste tot nu toe de toediening van afweeronderdrukkende medicijnen die te sterke bijwerkingen kennen om veilig te kunnen worden toegepast in patiënten. De doelen van het onderzoek waren (i) het vergroten van het begrip van de ontstekingsreactie die optreedt als menselijke en varkenseilandjes in direct contact komen met de bloedbaan, (ii) het voorkomen van deze ontstekingsreactie en humorale afstoting door het transplanteren van eilandjes van varkens die een menselijk gen voor complement-regulatie tot expressie brengen, (iii) het onderzoeken van andere anatomische locaties dan de lever als locatie voor eilandjestransplantatie en (iv) het onderzoeken van veiliger strategieën voor afweeronderdrukking.

Hoofdstuk 2 beschrijft de resultaten van een literatuurstudie naar factoren die bijdragen aan de ontstekingsreactie na eilandjestransplantatie in de poortader. De ontstekingsreactie bestaat uit activering van stolling en complement, plaatjesaggregatie en infiltratie van neutrofiele granulocyten en macrofagen en treedt op na zowel allotransplantatie als xenotransplantatie. De ontstekingsreactie, met als gevolg het verlies van eilandjes, zal geminimaliseerd dienen te worden om effectiever gebruik te maken van het reeds beperkte aantal menselijke donororganen. Ook in xenotransplantatie, waarbij het aantal varkenseilandjes theoretisch ongelimiteerd is, is effectieve bestrijding van dit eilandjesverlies wenselijk indien het doel is een praktisch toepasbare therapie te ontwikkelen. Strategieën ter bestrijding van de ontstekingsreactie worden beschreven.

Hoofdstuk 3 beschrijft hoe een *in vitro* model voor de ontstekingsreactie werd opgezet waarin menselijke en varkenseilandjes werden blootgesteld aan menselijk bloed. Hoewel menselijke en varkenseilandjes in gelijke mate

stolling van menselijk bloed veroorzaakten, was de celschade toegebracht aan varkenseilandjes significant groter. Dit verschil werd mogelijk veroorzaakt door antilichamen gericht tegen varkenseilandjes die activering van het complementsysteem induceerden. Celschade aan varkenseilandjes trad eveneens op na blootstelling aan menselijk plasma en kon deels worden voorkomen door specifieke blokkade van antilichamen en complement. Deze resultaten geven het belang van de humorale afweer in de ontstekingsreactie na eilandjestransplantatie in de poortader aan.

In **Hoofdstuk 4** werden diabete apen met varkenseilandjes getransplanteerd. Er werden varkensdonoren gebruikt die het menselijke gen voor CD46 (hCD46), een complement-regulerend gen, transgenetisch tot expressie brengen op cellen in de eilandjes. Apen die varkenseilandjes zonder hCD46 kregen getransplanteerd, vormden de controlegroep. De transplantatie van eilandjes van hCD46-transgene varkens resulteerde in normale bloedsuikerwaarden zonder insuline-injecties in vier van vijf apen voor de volledige duur van het experiment (3 maanden). Eén van deze apen werd langer dan een jaar gevolgd en bleef genezen van diabetes tot het experiment werd gestaakt. Deze resultaten waren significant beter dan in de controlegroep. Het blokkeren van complementactivering door hCD46 resulteerde echter niet in het voorkomen van de ontstekingsreactie. Daarentegen leidde het tot preventie van afstoting door circulerende antilichamen tegen varkensantigenen, waardoor afstoting met minder afweeronderdrukkende medicatie dan voorheen kon worden voorkomen.

Omdat het volume van een eilandjestransplantaat slechts één tot twee milliliter bedraagt, zouden de eilandjes relatief eenvoudig op andere plaatsen in het lichaam kunnen worden geïnjecteerd. Verscheidene anatomische locaties zijn eerder in klinische en experimentele studies uitgeprobeerd en een literatuurstudie hiervan is beschreven in **Hoofdstuk 5**. Vervolgens worden in **Hoofdstuk 6** de resultaten gerapporteerd van eilandjestransplantatie in de maagwand van varkens met diabetes. Het bleek mogelijk de transplantatie in de maagwand uit te voeren middels een kortdurend kijkonderzoek via de mond en de slokdarm (endoscopie). Een operatie was niet nodig en de ingreep was vrij van complicaties. Doordat eilandjes niet direct in de bloedbaan werden getransplanteerd, werd de ontstekingsreactie voorkomen.

Het doel van **Hoofdstuk 7** was om alemtuzumab (monoklonale antilichamen tegen CD52) toe te passen in apen. Alemtuzumab is een zeer effectief medicijn gebleken voor depletie van lymfocyten in patiënten die een transplantatie ondergaan. Echter, de meeste apensoorten brengen CD52 ook tot expressie op rode bloedcellen en het toedienen van alemtuzumab zou ernstige hemolyse kunnen veroorzaken. Met een agglutinatie-screeningsmethode konden cynomolgus makaken worden geïdentificeerd die negatief waren voor CD52-expressie op rode bloedcellen. Alemtuzumab in hoge dosering kon vervolgens veilig in deze

apen worden toegepast, en leidde tot bijna complete lymfodepletie in het bloed en in perifere lymfeklieren. Deze depletie was significant groter dan in apen die anti-thymocyte globuline kregen toegediend. De T lymfocyten die door de tijd in het bloed verschenen hadden met name het phenotype van geheugen T lymfocyten. Dit preklinische model biedt mogelijkheden om het lymfocyten depletie-repopulatie fenomeen te onderzoeken en een afweeronderdrukkend medicijnprotocol te ontwikkelen dat effectief afstoting van het xeno-eilandjestransplantaat voorkomt en tegelijkertijd veilig toepasbaar is in patiënten.

In **Hoofdstuk 8** werd de ontwikkeling van het immuunsysteem van pasgeboren bavianen gevolgd tot ze de leeftijd van 2,5 jaar bereikten. Het doel was om twee fundamentele immunologische principes die eerder zijn geobserveerd in laboratoriummuizen - namelijk neonatale lymfopenie-geïnduceerde proliferatie van geheugen T cellen en mogelijkheden voor inductie van tolerantie voor transplantaatantigenen - te verifiëren in een model dat gebruik maakt van primaten. We observeerden dat bavianen bij de geboorte een lymfopeen en naïef immuunsysteem hebben en vervolgens het aantal T cellen uitbreiden middels de expansie van geheugencellen. Op de leeftijd van drie maanden werden bavianen getransplanteerd met een allo- of xeno-arteriesegment (als een model voor harttransplantatie), met of zonder toediening van afweeronderdrukkende medicijnen. Zonder onderdrukking van de afweer trad afstoting op. Aanwijzingen voor tolerantie-inductie werden niet waargenomen. Afstoting werd effectief voorkomen met immunosuppressie gericht op T lymfocytdepletie en cellulaire afstoting. Na lymfodepletie werd homeostatische proliferatie van geheugen T cellen waargenomen. Geheugen T cellen kunnen een barrière vormen voor de inductie van transplantaattolerantie of het afbouwen van afweeronderdrukkende medicatie.

Hoofdstuk 9 geeft een interpretatie en discussie van de studies in dit proefschrift. Ook worden methodologie en toekomstperspectieven bediscussieerd.

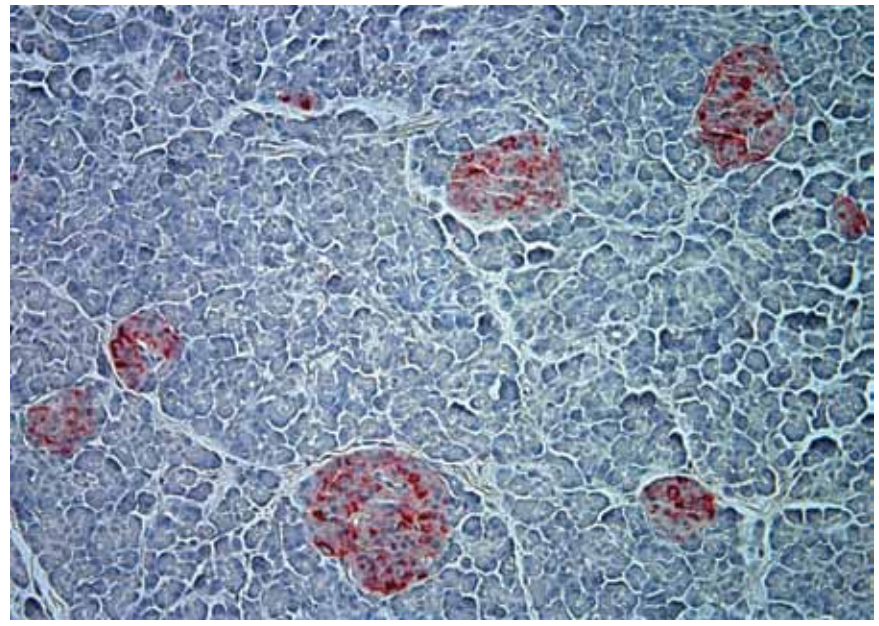
APPENDICES

List of Abbreviations

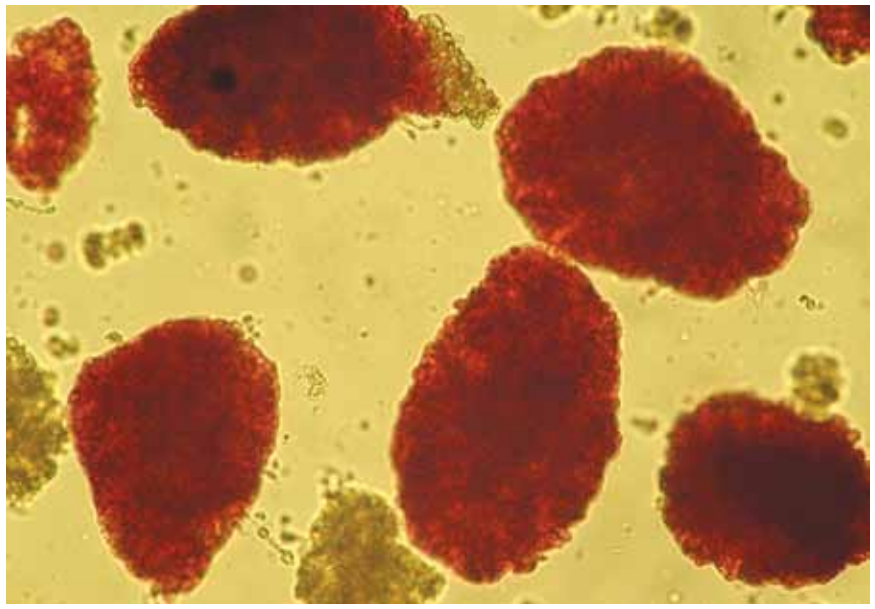
AB-I ..... ABO-incompatible
ACR ..... acute C-peptide response
alloTx ..... allotransplantation
AST ..... arginine stimulation test
ATG ..... anti-thymocyte globulin
autoTx ..... autotransplantation
BSA ..... bovine serum albumin
CRP ..... complement-regulatory protein
EDTA ..... ethylenediamine tetraacetic acid
ELISA ..... enzyme-linked immunosorbent assay
Gal galactoseα1,3galactose
GT-KO ..... α1,3-galactosyltransferase gene-knockout
GSMS ..... gastric submucosal space
hCD46 ..... human CD46 transgenic
H&E ..... hematoxylin and eosin
HLA ..... human leukocyte antigen
IBMIR ..... instant blood-mediated inflammatory reaction
IEQ ..... islet equivalents
IS ..... immunosuppressive therapy
IVGTT ..... intravenous glucose tolerance test
LIP ..... lymphopenia-induced proliferation
LMW-DS ..... low molecular weight dextran sulfate
mAb ..... monoclonal antibody
MAC ..... membrane-attack complex
MCP-1 ..... monocyte chemoattractant protein-1
MLR ..... mixed lymphocyte reaction
MMF ..... mycophenolate mofetil
NAC ..... nacystelyn
NHP ..... non-human primate
NK cells ..... natural killer cells
PBMC ..... peripheral blood mononuclear cells
PERV ..... porcine endogenous retrovirus
PI ..... propidium iodide
RBC ..... red blood cells

RIA ..... radioimmunoassay
SI ..... stimulation index
STZ ..... streptozotocin
T1D ..... type 1 diabetes
T_{CM} ..... central memory T cells
T_{EM} ..... effector memory T cells
T_{EMRA} ..... terminally differentiated effector memory T cells
TF ..... tissue factor
Tg ..... transgenic
T_{N} ..... naïve T cells
T_{Reg} ..... regulatory T cells
T_{TotMem} ..... total memory T cells
Tx ..... transplantation
vWf ..... von Willebrand factor
WBC ..... white blood cells
WT ..... wild-type
xenoTx ..... xenotransplantation

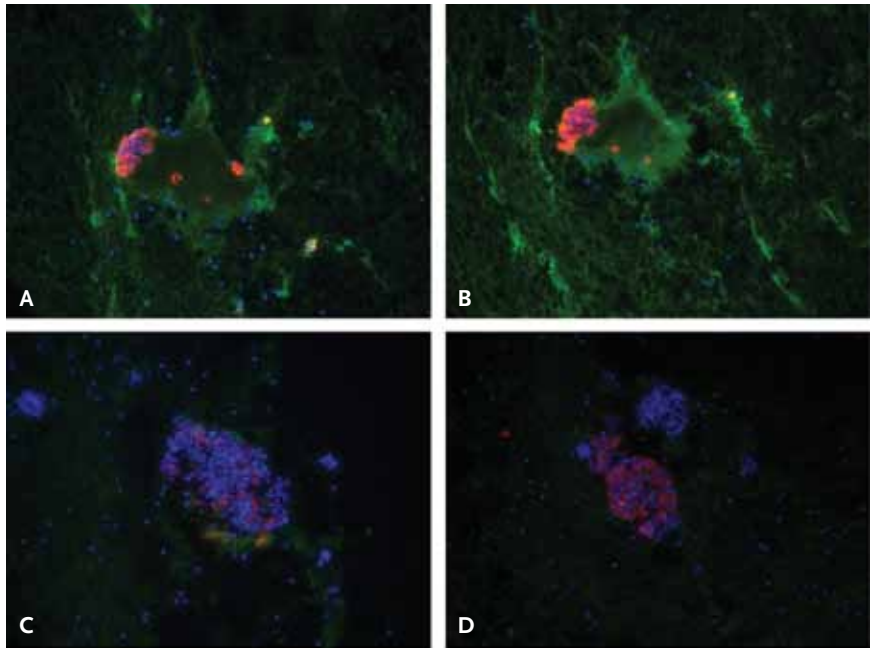
Color Figures



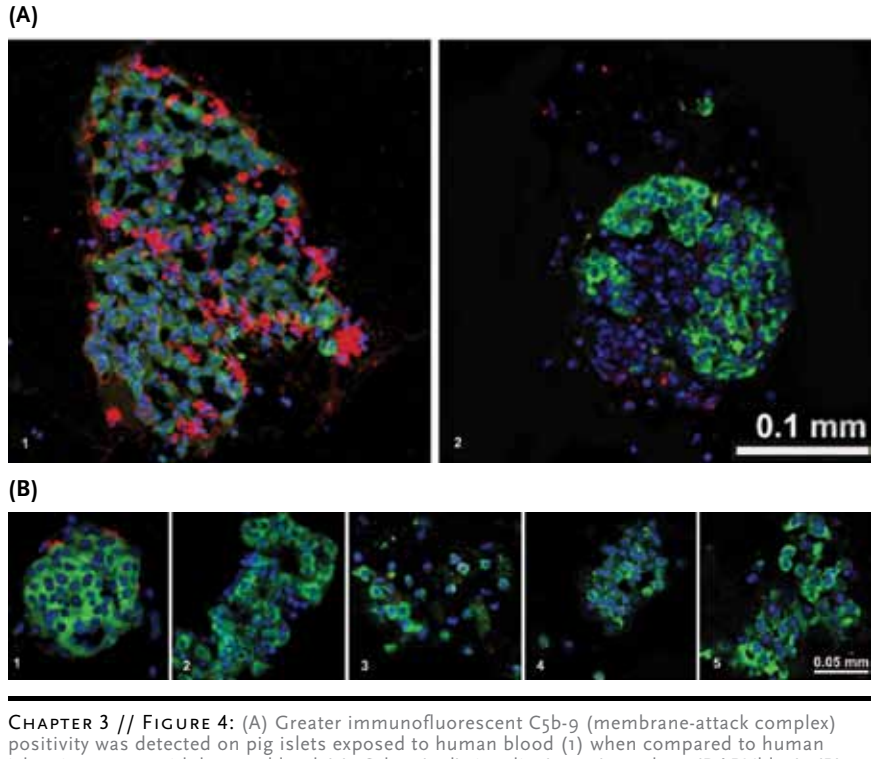
Chapter 1 // Figure 1: Light microscopy image (40x) of human pancreas showing islets, immunostained for insulin (red).



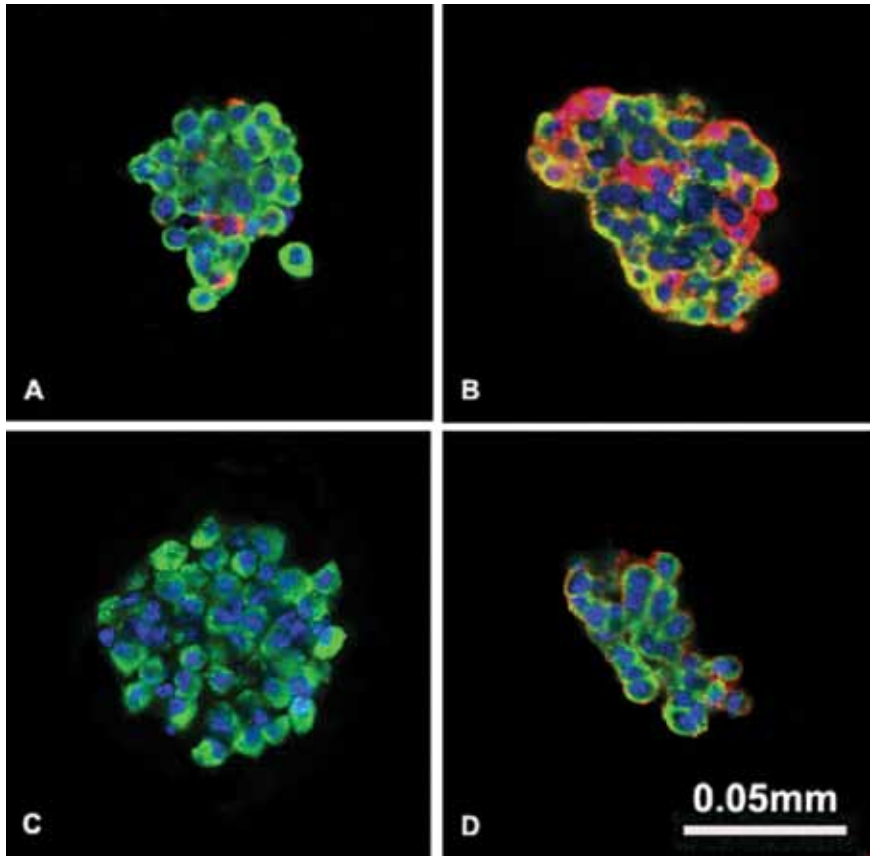
CHAPTER 1 // FIGURE 2: Human islets after isolation and purification, stained *in vitro* with dithizone (red).



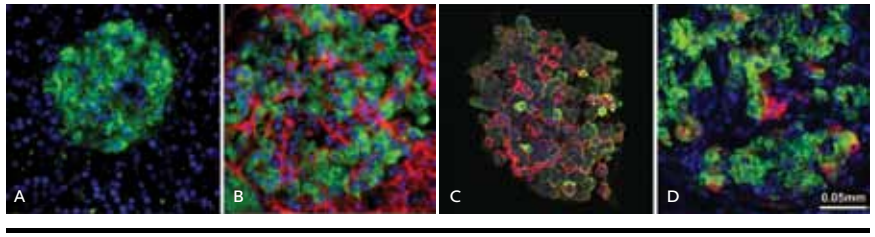
CHAPTER 3 // FIGURE 2: Binding of human IgM and IgG antibody to pig islets (xenogeneic) (A-B), and to human islets (allogeneic) (C-D). IgM (green, A,C), IgG (green, B,D), insulin (red), nucleus (DAPI/blue). Yellow indicates co-localization of insulin and IgM/IgG.



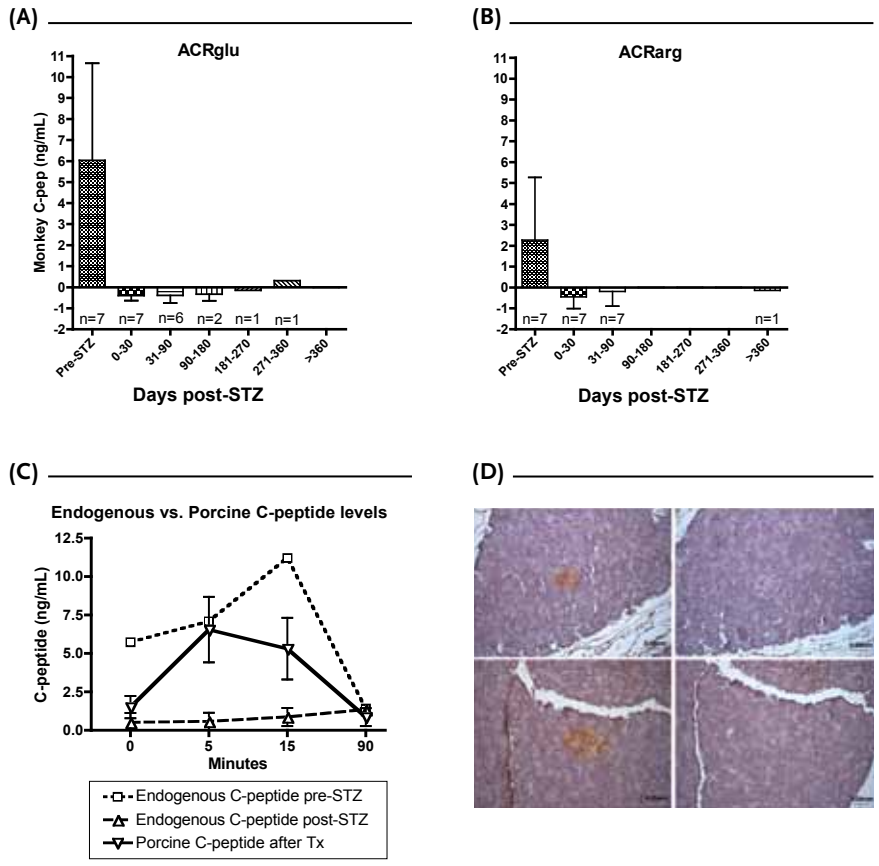
CHAPTER 3 // FIGURE 4: (A) Greater immunofluorescent C5b-9 (membrane-attack complex) positivity was detected on pig islets exposed to human blood (1) when compared to human islets in contact with human blood (2). C5b-9 (red), insulin (green), nucleus (DAPI/blue). (B) C5b-9 staining on pig islets after treatment with: LMW-DS (1), NAC (2), compstatin (3) and anti-IgM (4). hCD46 transgenic pig islet (5). C5b-9 (red), insulin (green), nucleus (DAPI/blue).



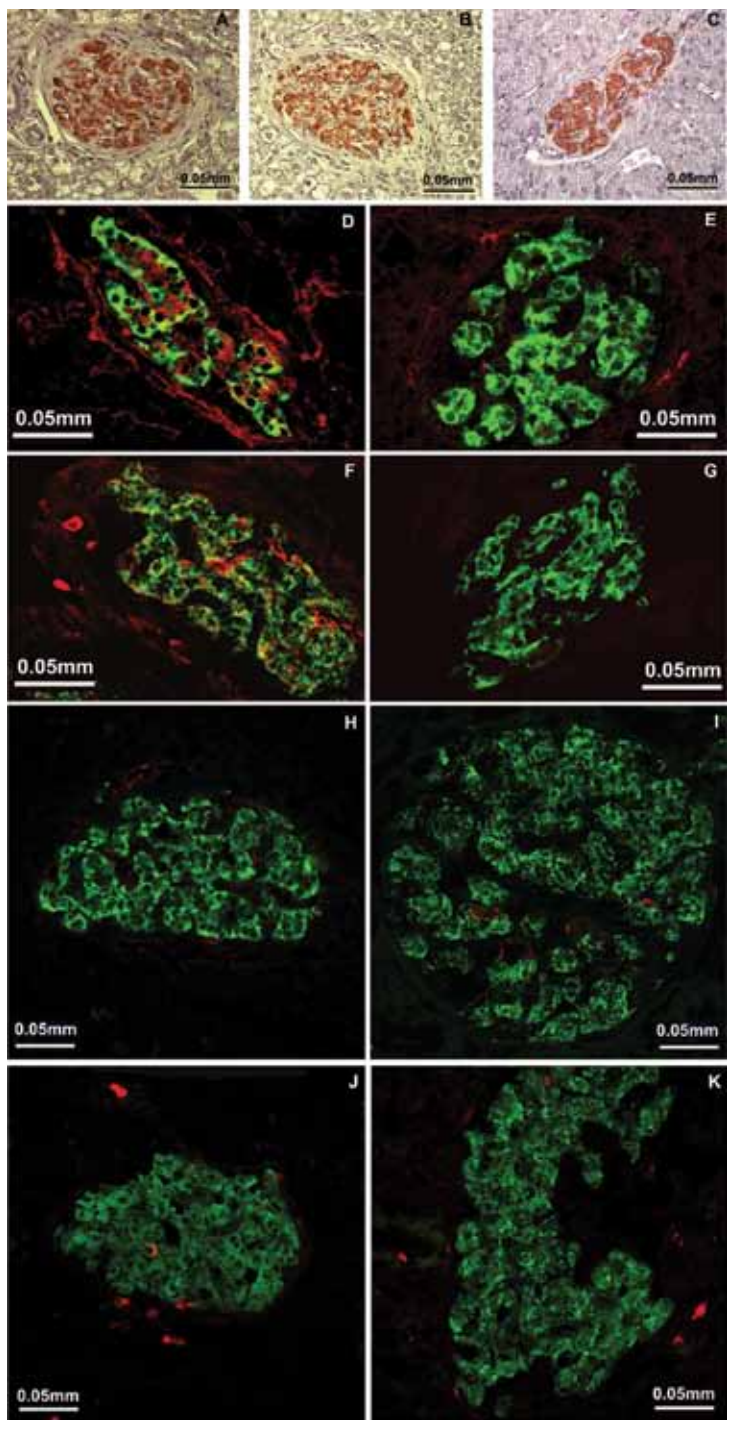
CHAPTER 3 // FIGURE 8: C5b-9 and IgM deposition after exposure of pig islets to human plasma (A-B) and to heat-inactivated plasma (C-D). C5b-9 (red, A and C), IgM (red, B and D), insulin (green), nucleus (DAPI/blue).



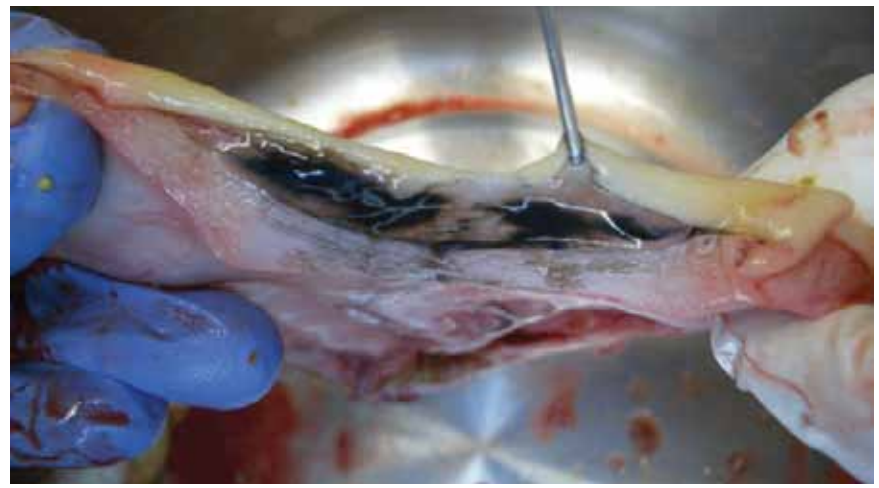
CHAPTER 4 // FIGURE 1 | EXPRESSION OF HCD46: Anti-insulin (green) and anti-hCD46 (red) antibody staining of pig pancreatic tissue and islets. (A) WT pig pancreas (Group A); (B) hCD46 transgenic pig pancreas (Group B). (C) Isolated hCD46 transgenic pig pancreatic islet prior to transplantation; and (D) islet in the liver of the monkey recipient one year after transplantation (Group B).



CHAPTER 4 // FIGURE 3 | DIABETES INDUCTION BY I.V. STREPTOZOTOCIN: Absence of an endogenous acute C-peptide response (ACR) after induction of diabetes in monkey recipients, when metabolically challenged with (A) intravenous glucose (glu) or (B) arginine (arg). (C) Endogenous and porcine C-peptide levels during IVGTT in Monkey 7273, the islet recipient followed-up for over one year. Data points are mean ± SD of N=7 tests. The last test was carried out three days prior to euthanasia. (D) Left sets of images: glucagon immunostaining of pancreatic tissue analysed in Monkey 7273, right panels show insulin immunostaining of consecutive sections.

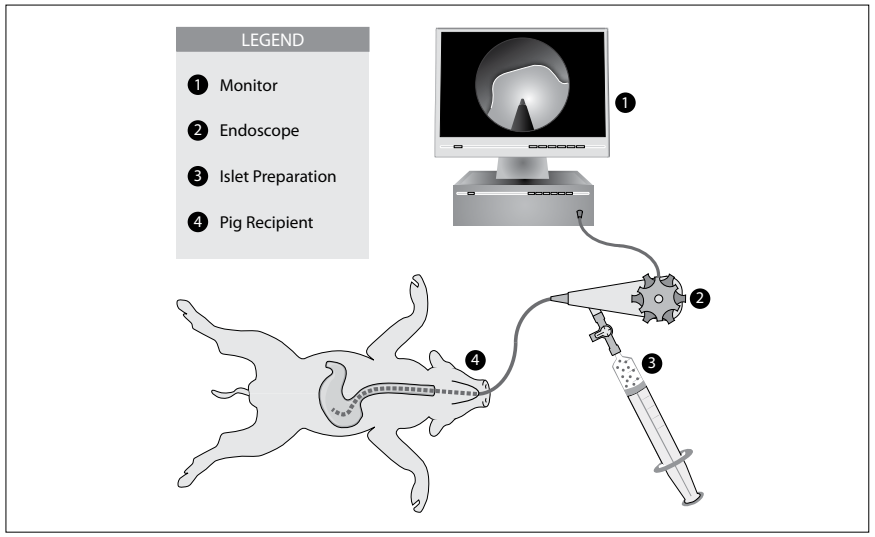


CHAPTER 4 // FIGURE 6 | POST-TRANSPLANT HISTOLOGY: (A-C) Insulin immunostaining of liver sections in three monkey recipients after pig hCD46 islet transplantation. (A and B) M29166 and M6075 at three months after transplantation; (C) M7273 >1 year after transplantation. (D-K) Immunofluorescence staining of the liver of monkey recipients following islet transplantation. In all panels insulin is stained green. IgG staining (red) of an islet from a monkey recipient of WT pig islets (Group A) (D) and hCD46 pig islets (Group B) (E). C4d staining (red) of an islet from a monkey recipient of WT pig islets (Group A) (F) and hCD46 pig islets (Group B) (G). Macrophage immunostaining and CD97 positive cells in Group A (respectively H,J) and in Group B (I,K).

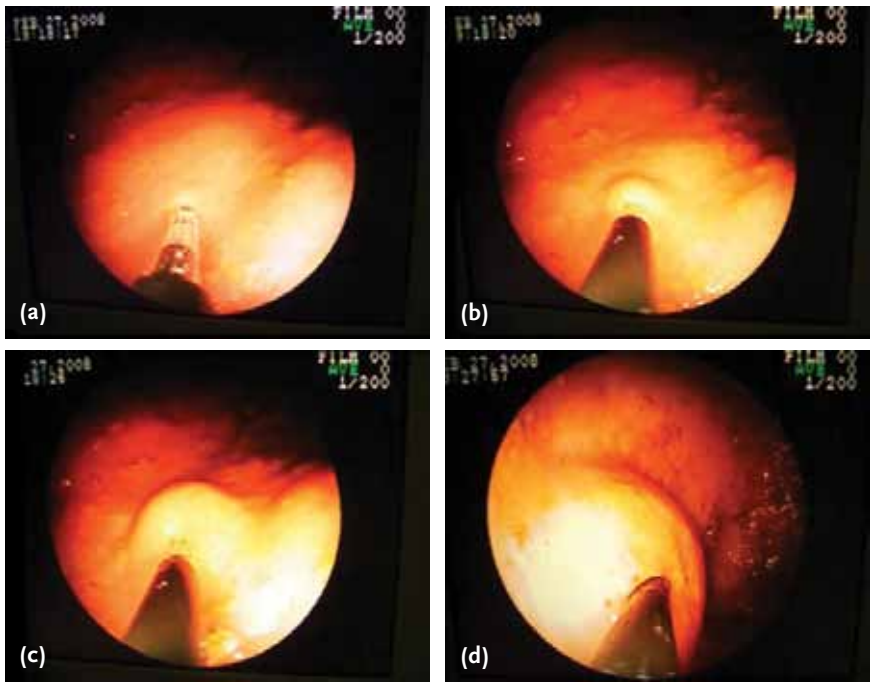


CHAPTER 6 // FIGURE 1: Macroscopic appearance of stomach wall after endoscopic injection of ink into the GSMS (as described in the text).

(A)

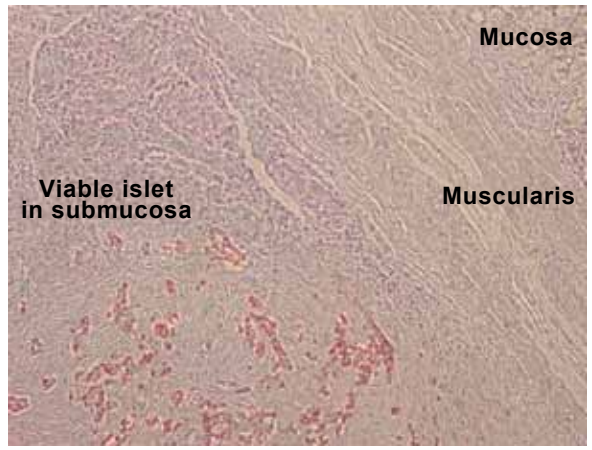


(B)

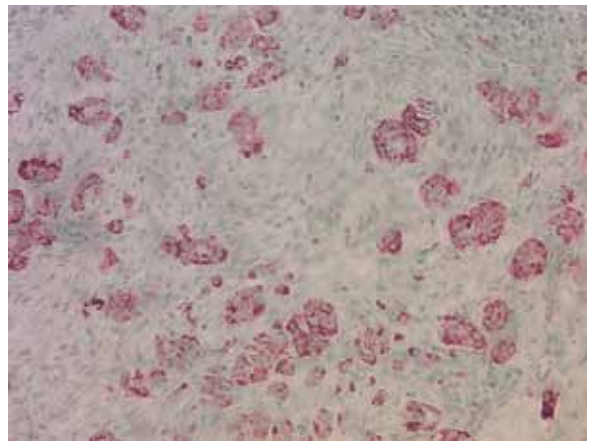


CHAPTER 6 // FIGURE 2: Endoscopic procedure of islet Tx into the GSMS (ENDO-STI). (A) Diagram of the procedure. (B) Endoscopic views during the procedure. (a) the endoscope needle is exposed, (b) inserted through the mucosa of the anterior gastric antrum at an approximate 45 degree angle, (c) the islets begin to be injected, and (d) the injection is completed. The procedure is then repeated at other sites.

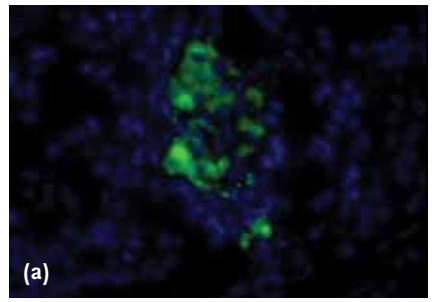
(A)

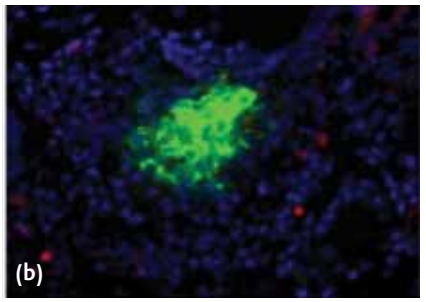


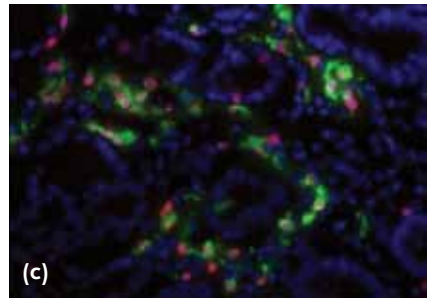
(B)



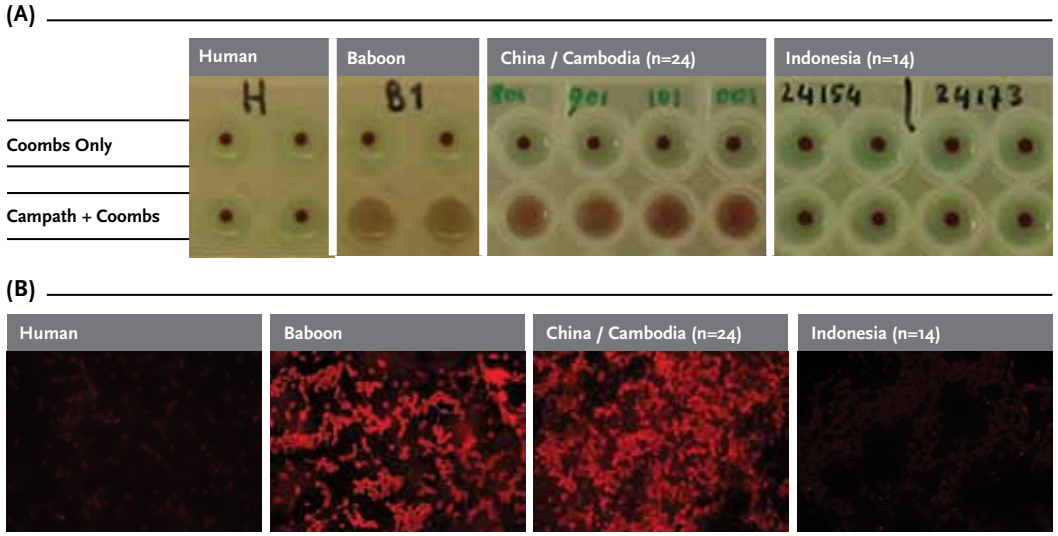
(C)



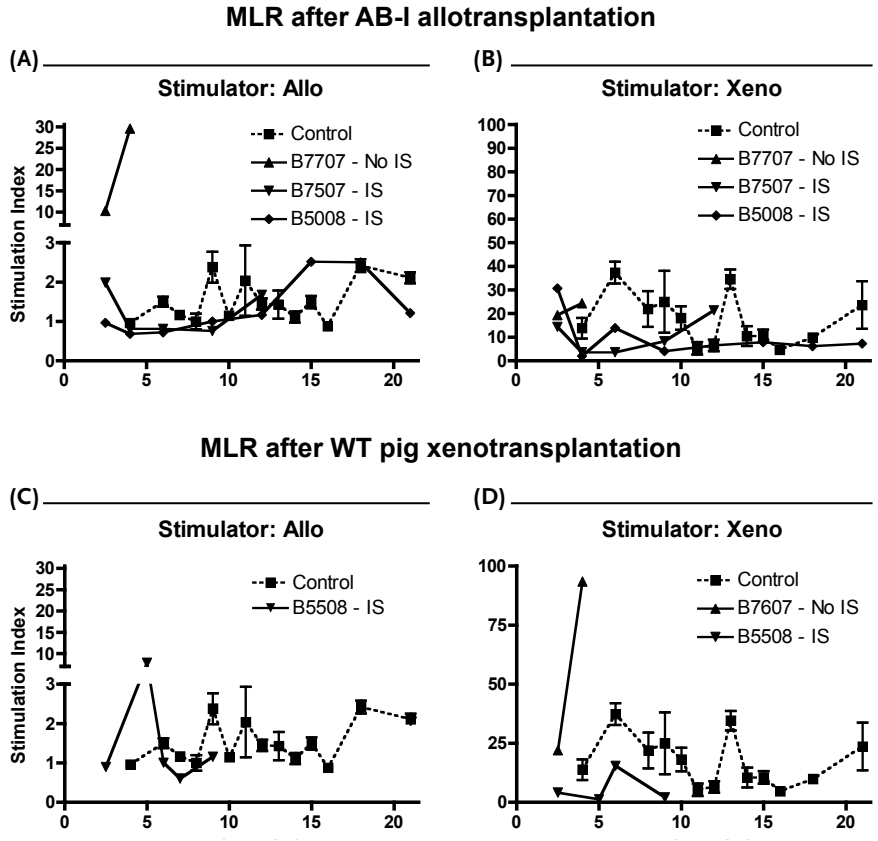


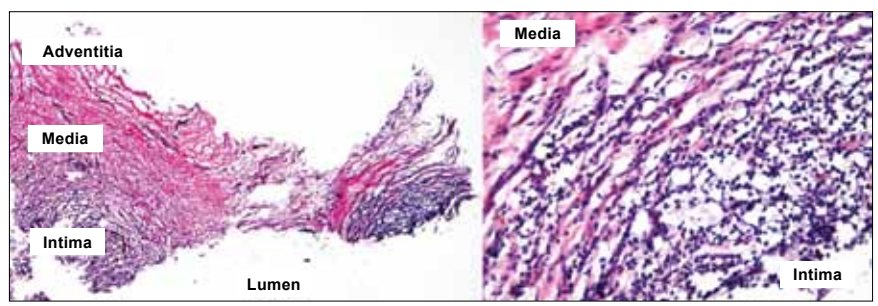


CHAPTER 6 // FIGURE 5: Histological appearances of the gastric wall following islet Tx into the GSMS in the Group 1 pigs. (A) Mucosa (upper right), muscularis mucosa, and GSMS (lower left), with viable insulin-staining of multiple islets (lower left) on day 14 after islet Tx (Pig #03308; H&E and insulin immunohistochemistry, magnification x10). (B) Healthy-looking islets 28 days after Tx, with a mild mononuclear cell infiltrate (Pig #07908; H&E and Insulin immunohistochemistry, x20). (C) Immunofluorescence staining for presence of apoptosis (red - TUNEL; green - insulin). (a) No TUNEL-staining was seen 24h after islet Tx, indicating an absence of apoptosis (Pig #00208). Late in the post-Tx period, e.g., 21-28 days, the incidence of apoptosis varied, being absent in some cases (b) (Pig #03408), but present in others (c) (Pig #35907).



CHAPTER 7 // FIGURE 1 | CD52 EXPRESSION ON RED BLOOD CELLS (RBC): (A) Representative results of the agglutination screening test. Non-agglutinated RBC were spun towards one point in the round-bottom wells. In contrast, wells with agglutinated cells had a cloudy appearance as a result of the RBC being fixed to one another by alemtuzumab and secondary antibody. (B) Immunofluorescent staining of RBC confirmed the agglutination results, showing bright staining for CD52 on all baboon and Chinese and Cambodian monkey RBC. Staining was completely absent on human and Indonesian monkey RBC.





CHAPTER 8 // FIGURE 4 | STIMULATION INDEX AFTER MIXED LYMPHOCYTE REACTION (MLR) AND HISTOLOGY IN TRANSPLANTED (GROUP 2) BABOONS: Responses in healthy infant baboons (Control) are compared with responses in baboons after AB-I allo Tx (A, B) and WT pig xeno Tx (C, D) in the presence (IS) or absence (No IS) of immunosuppressive therapy. Data for B7607 after allo-stimulation are missing due to technical error (C). (E) Graft histology 6 weeks after Tx without IS (Group 2A). Left panel (magnified 4x) shows loss of architecture. The location of tunica media in the vessel wall can still be recognized by collagen and smooth muscle (pink), but vast cellular infiltration has caused damage throughout the vessel wall (B7607). Right panel: lymphocytic infiltration of intima and tunica media (magnified 40x, B7607). Clear (transparent) spaces are artifacts due to suboptimal tissue storage.

Acknowledgements / Dankwoord

Dit promotietraject is een levensfase gebleken waarin ik zowel een wetenschappelijke als persoonlijke ontwikkeling heb doorgemaakt; ik zou niet kunnen zeggen welke groter is geweest. Onderweg trok ik op met veel behulpzame, interessante, geleerde, hardwerkende, getalenteerde, geïnteresseerde, warme en lieve mensen die allen een bijdrage leverden aan deze ontwikkelingen. Ik ben hen allen zeer dankbaar. Een aantal mensen wil ik hier in het bijzonder noemen.

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List of Publications

PUBLICATIONS RELATED TO THE RESEARCH OF THIS DISSERTATION:

van der Windt DJ, Bottino R, Casu A, Campanile N, Cooper DKC. Rapid loss of intraportally transplanted islets: an overview of pathophysiology and preventive strategies.
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van der Windt DJ, Smetanka C, Macedo C, He J, Lakomy R, Bottino R, Ekser B, Echeverri GJ, Metes D, IJzermans JNM, Trucco M, Cooper DKC*, Lakkis FG*. Investigation of lymphocyte depletion and repopulation using alemtuzumab (Campath-1H) in cynomolgus monkeys.
*Am J Transplant 2010; 10: 773-783. *contributed equally*

Ekser B, Ezzelarab M, Hara H, **van der Windt DJ**, Wijkstrom M, Bottino R, Trucco M, Cooper DKC. Clinical xenotransplantation: the next great medical revolution?
Submitted.

Dons EM, Montoya C, Hara H, Long C, Echeverri GJ, Ekser B, Ezzelarab C, Roa Medellin D, **van der Windt DJ**, Murase N, Rigatti L, Wagner R, Wolf RF, Ezzelarab M, West LJ, IJzermans JNM, Cooper DKC. T cell-based immunosuppressive therapy inhibits the development of natural anti-AB blood type and anti-pig antibodies in infant baboons.
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van der Windt DJ*, Marigliano M*, He J, Votyakova TV, Echeverri GJ, Ekser B, Ayares D, Lakkis FG, Cooper DKC, Trucco M, Bottino R. Early islet damage after direct exposure of pig islets to blood – has humoral immunity been underestimated?
*Submitted. *contributed equally*

van der Windt DJ*, Dons EM*, Montoya CL, Ezzelarab M, Long C, Wolf RF, IJzermans JNM, Lakkis FG, Cooper DKC. T-lymphocyte homeostasis and function in infant baboons: implications for transplantation.
*Submitted. *contributed equally*

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*AJR Am J Roentgenol 2008; 191: 1430-1435. *contributed equally*

van Aalten SM, Terkivatan T, de Man RA, **van der Windt DJ**, Kok NFM, Dwarkasing R, IJzermans JNM. Diagnosis and treatment of hepatocellular adenoma in the Netherlands: similarities and differences.
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Ekser B, Echeverri GJ, Hassett AC, Yazer MH, Long C, Meyer M, Ezzelarab M, Lin CC, Hara H, **van der Windt DJ**, Dons EM, Phelps C, Ayares D, Cooper DKC, Gridelli B. Hepatic function after genetically engineered pig liver transplantation in baboons.
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J Hepatol 2010; 54: 553-558.

PhD Portfolio

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Prof. David K.C. Cooper, MD, PhD

RESEARCH TRAINING

Courses attended to obtain the MSc degree in Clinical Epidemiology, Netherlands Institute for Health Sciences, Rotterdam, Netherlands, 2003-2005:

Erasmus University Rotterdam, Rotterdam, Netherlands
- Principles of Research in Medicine and Epidemiology
- Study Design
- Clinical Decision Analysis
- Methods of Clinical Research
- Topics in Evidence-based Medicine
- Methods of Health Services Research
- Prevention Research
- Introduction to Data-analysis
- Regression Analysis
- Survival Analysis
- Introduction to Clinical Research
- Pharmaco-epidemiology and Drug Safety
- Intervention Research and Clinical Trials
- Diagnostic Research
- Advances in Prognostic Research
- Topics in Meta-analysis
- Causal Modelling
- History of Epidemiologic Ideas
- Bayesian Statistics
- Advanced Topics in Clinical Trials
- Analysis of Time-varying Exposures

University of Cambridge, Cambridge, UK
- Diabetes Module of the MPhil in Epidemiology
- Cardiovascular Disease Epidemiology

Johns Hopkins Bloomberg School of Public Health, Baltimore, USA
- Intermediate Epidemiology
- Epidemiology in Evidence-based Decisions
- Social Epidemiology

ORAL PRESENTATIONS

Mechanisms of in vitro xenogeneic IBMIR: involvement of classical and alternative pathways of complement activation.
International Pancreas & Islet Transplant Association - International Xenotransplantation Association, Venice, Italy, 2009.

Transplantation of hCD46 transgenic porcine islets into diabetic nonhuman primates results in long-term normoglycemia under limited immunosuppression.
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About the Author

Dirk Jan van der Windt was born March 1st 1983 in Rotterdam, Netherlands. He attended the Sint-Laurenscollege in Rotterdam from 1995-2001. In 2001 he started medical school at the Erasmus University Rotterdam. His research career started when he was accepted to the extracurricular program of the Netherlands Institute for Health Sciences in 2003, from which he graduated in 2005 with a M.Sc. degree in Clinical Epidemiology. During this program he conducted his first research studies regarding hepatocellular adenoma, a benign liver tumor (Prof.dr. Jan N.M. IJzermans). In 2006, he interrupted his medical studies to investigate islet xenotransplantation at the Children's Hospital of Pittsburgh (Prof.dr. Massimo Trucco) and the Thomas E. Starzl Transplantation Institute (Prof.dr. David K.C. Cooper), both at the University of Pittsburgh, USA. This dissertation is based on studies carried out at the University of Pittsburgh. He resumed his clinical training in 2009 and graduated with his M.D. degree in 2010. He hopes to continue his research efforts and to begin a residency program in general surgery. Dirk enjoys running and will again participate in the New York City marathon this year, together with his two sisters and his father.

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