ACUTE ANTIBODY-MEDIATED REJECTION IN PANCREAS AND KIDNEY TRANSPLANTATION

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Acute antibody-mediated rejection in pancreas and kidney transplantation
Dissertation, University of Leiden, Leiden, the Netherlands

ISBN/EAN: 978-94-6182-189-8

Cover design, lay-out and print by: Off page, www.offpage.nl

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The studies reported in this thesis were performed at the department of Pathology (head: Prof. Dr. G.J. Fleuren) and the department of Nephrology (head: Prof. Dr. A.J. Rabelink), Leiden University Medical Center, Leiden, the Netherlands.

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Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties

te verdedigen op donderdag 7 februari 2013
klokke 15.00 uur

door

Hanneke de Kort

geboren te Tilburg
in 1983

PROMOTIECOMMISSIE

Promotores:	Prof. Dr. J.A. Bruijn Prof. Dr. J.W. de Fijter
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Part of the research described in this thesis was financially supported by a collaboration of the Juvenile Diabetes Research Foundation international, the Dutch Diabetes Research Foundation Netherlands, and the Netherlands Organization for Health Research and Development (2003.10.002).

Financial support for publication of this thesis by the department of Pathology, the Waleaus library, de Nederlandse Transplantatie Vereniging, Astellas Pharma BV, het Diabetes Fonds, Novartis Pharma BV, Roche Nederland BV, Novo Nordisk BV, and Sakura Finetek Europe BV is gratefully acknowledged.

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1 | INTRODUCTION

1. KIDNEY TRANSPLANTATION

The first successful kidney transplantation was performed in 1954[1], and renal transplantation has since been the most studied organ transplant procedure. At present, approximately 28,500 kidney transplantations are performed annually in the US (based on Organ Procurement and Transplantation Network data as of March 2, 2012). With the improvements in surgical techniques, better immunosuppressive drugs and regimen refinements, acute rejection rates have dropped from 80% to a mere 10-20% in current daily practice[2]. However, despite the improved one-year graft survival rates[2;3], long-term outcomes have not improved concomitantly in the last decade, and beyond the first year after transplantation, graft attrition rates remain between 3 and 5% annually[4].

There are several factors that may hamper the long-term success of kidney transplantation, including both immunological (acute and/or chronic rejection) and non-immunological (such as drug-related nephrotoxicity, long-term effects of ischemia-reperfusion injury, hyperlipidemia and hypertension)[5] causes. Acute rejection is caused by T-cell- and/or antibody-mediated processes. The focus of the studies described in this thesis is on antibody-mediated rejection, which was investigated in both the kidney and pancreas transplantation setting. Furthermore, the effect of cellular immune modulation was investigated in an allogeneic rodent islet transplantation model.

This chapter provides the reader with an introduction to these topics, starting with a general description of transplant rejection and a more detailed overview of antibody-mediated rejection. Type 1 diabetes, an increasingly prevalent cause of end-stage renal failure, will be introduced as well as the currently available potentially curative therapies, including whole pancreas and islets of Langerhans transplantation.

1.1 Rejection in transplantation

Acute antibody-mediated rejection is the main focus of this thesis, concentrating on kidney, pancreas and islets of Langerhans transplantation. Regardless of the transplanted tissue, every allograft may be faced with the consequences of the activation of the host's defense immune system. The immune system specializes in distinguishing self from non-self, and as such, it can eliminate infectious agents and reduce tissue damage. First, the innate immune system tries to fend off the non-self donor tissue, after which the acquired immunity starts its course of action. Both these first and second lines of defense may result in the rejection of the allograft. Therefore, immunosuppressive therapy should preferably be introduced well before the transplant procedure. However, acute rejection episodes occur even in the setting of pre-dosed live donor transplants, underscoring the fact that immunosuppressive drugs have their inherent limitations.

In transplantation, there are two immunological pathways through which rejection can occur: T-cell-mediated cellular rejection (TCR) and antibody-mediated rejection (AMR)[6]. Both types of rejection can occur individually or simultaneously, and as such, they can be difficult to discriminate. Distinguishing between these two processes is essential as treatment for each type of rejection differs. The treatment of an acute T-cell-mediated rejection

episode includes the use of high-dose corticosteroids and/or (T-cell depleting) antibody therapy such as polyclonal anti-thymocyte globulin or monoclonal anti-CD3 antibodies[7]. Treatment for AMR is aimed at removing or depleting donor-specific antibodies (DSA) from the recipient's circulation through intravenous immunoglobulins, plasmapheresis, anti-CD20 antibody therapy and off-label use of therapies that are still under investigation, such as eculizumab (anti-C5 therapy) and bortezomib (proteasome inhibition)[8].

Allograft rejection, both T-cell and antibody-mediated, is currently classified according to histological grading schemes defined and updated at regular Banff conferences on allograft pathology. The first meeting was held in Banff, Canada, in 1991 and resulted in the first published Banff scoring schema in 1993[9]. In subsequent years, the hallmarks of TCR have been classified in such schemes for solid organ transplants, including kidney, liver, pancreas and skin-containing composite tissue[10-13]. The extent and type of the lesions affects therapy regimens, considering that minor tissue damage can be resolved with a short course of high-dose steroids. More severe or extensive damage, however, must be dealt with more rigorously, or the graft will be lost. Several studies have increased our understanding of T-cells and their role in allograft recognition and rejection[14]. Thus our understanding of the mechanisms behind TCR has improved and has resulted in alternative treatment options. We have, however, also learned that none of the current drug therapies or regimens successfully eradicates both the effector and memory T-cell clones[14].

Thus, TCR has been well defined in solid organ transplantation, and current immunosuppressive regimens are primarily aimed at preventing this type of acute rejection. The typical histopathological and clinical findings in AMR are less well-defined. AMR may occur hyperacutely (within hours or days) or acutely (within weeks, months or even years) or may follow a more insidious chronic course. Antibody formation and the subsequent chronic damage, especially the chronic phase in which the least improvement has been made in graft survival, are currently the subject of several ongoing studies.

Antibody-mediated rejection

Antibody-mediated rejection occurs through distinct pathways. These pathways have in common the antibody-antigen interaction at the onset of endothelial damage. The antibodies responsible for these interactions are donor-specific antibodies (DSA), which are most often directed at human leukocyte antigen (HLA) but may also include minor histocompatibility antigens[15], endothelial cell specific antigens such as the major histocompatibility complex class I chain-related genes A and B (MICA or MICB)[16], glutathione-S-transferase T1[17] and transplanted antigens that have not yet been elucidated. This interaction may lead to complement-mediated damage through cellular lysis via the formation of the membrane attack complex C5b-9 or recruitment of inflammatory cells by specific soluble complement degradation fragments, such as C5a. The antibody-antigen interaction may also result in direct damage through the interaction with Fc-receptors on natural killer (NK) cells and NK-like T-cells, a process known as antibody-dependent cellular cytotoxicity (ADCC)[18].

Not all antibody-antigen interactions lead to rejection. Approximately 14-23% of all transplantation recipients with stable, functioning grafts have detectable anti-HLA antibodies[19;20]. Although graft survival is poorer in the anti-HLA-positive group, many transplant recipients retain graft function. These antibody-antigen interactions supposedly initiate damage repair by the endothelium, and as these antibodies persist, the continuous tissue repair process with accumulating damage results in a chronic antibody-mediated allograft rejection[21].

The importance of AMR has long been overlooked, and specific markers for this process have proven hard to find. A consensus was eventually reached for the kidney transplantation setting, stating that the presence of circulating DSA, certain histopathological findings in the biopsy and the deposition of C4d in the peritubular capillaries are the hallmarks of an ongoing AMR. The diagnostic relevance and therapeutic consequences of positive C4d staining has been investigated extensively in relation to kidney transplantation[22;23]. Complement factor 4 (C4) plays a central role in the activation cascade of the classical pathway of the complement system. Prior to secretion, the single-chain precursor is enzymatically cleaved to yield the non-identical chains α, ß, and γ. During activation, the α-chain is cleaved by C1 into C4a and C4b, while C4b stays linked to the ß and γ chains. Further degradation of C4b by C1 into the inactive fragments C4c and C4d blocks the generation of C3 convertase. The activated cascade leads to C5a production, which is a chemoattractant for several immune cells. Eventually, the membrane attack complex C5b-9 is formed, which can lead to permeabilization and subsequent lysis of the target cell. Thus, C4d is an inactive complement degradation product that is covalently bound to the site of action and can be used as a relatively long-lasting 'footprint' of the classical pathway activation of the complement cascade. However, C4d will also be deposited when the lectin pathway of the complement system is activated, given that it uses the same C3 convertase as the classical pathway. Recent studies have found that high MBL levels before transplantation are associated with inferior graft survival after renal and simultaneous pancreas-kidney transplantation[24;25]. This has not, however, been studied in conjunction with C4d deposition.

For kidney, heart, lung and small bowel transplantations, the relation between histopathological findings, the formation of antibodies after transplantation and C4d staining patterns have been previously addressed[26]. Initially, only diffuse C4d positivity of the peritubular capillaries (PTC) was considered clinically significant[27] and incorporated into the revision of the Banff '97 working classification of renal allograft pathology[28;29]. Since the Banff '07 classification of renal allograft pathology, the staining of more than 50% of PTC remains the standard for a truly positive staining, but the clinical significance of focal C4d staining (10-50% of PTC) is still a matter of debate[30]. The Banff '07 publication warrants a prospective case-control study with long-term follow-up in which C4d cutoffs are clearly defined, and simultaneous alloantibody detection is employed[30]. Recently, C4d-negative AMR has been described in the renal transplant setting, which can be distinguished through mRNA analyses of endothelial activation transcripts[31]. On

the other hand, in small bowel transplantation, the relationship between positive C4d staining and AMR is less pronounced than in kidney transplantation[32].

The role of AMR in pancreas transplantation has not been extensively studied. A search of the literature up to April 2008 (the date of the first publication of the Banff classification scheme for pancreas allograft rejection) reveals 2 case reports of patients with biopsy-proven AMR of the pancreas[33;34] and one study of 136 patients with SPKT, in which 2 pancreas transplant biopsies were available that were both positive for C4d[35]. In the first Banff classification of pancreas allograft rejection[36], C4d was included as a marker for AMR. At that time, however, only a few case reports had documented C4d in pancreas transplant biopsies. After April 2008, larger studies were performed to assess the role of AMR in pancreas transplantation. Torrealba *et al* were the first to describe C4d staining patterns in pancreas transplants and reported that only the positive staining of interacinar capillaries (IAC) was of significance. Furthermore, the authors found that the C4d staining of IAC correlated with circulating donor-specific antibodies[37]. To date, four other relatively large studies on the relevance of DSA and AMR in pancreas transplantation have been performed[38-41]. In the latest Banff classification of pancreas allograft rejection[42], the same characteristics have been defined for AMR as in the kidney transplant setting. This definition has resulted in the exclusion of graft dysfunction[36] as a parameter and the incorporation of histological evidence of graft injury. Evidently, additional research is necessary for the pancreas transplant setting and is addressed in the work described in this thesis.

2. TYPE 1 DIABETES AND TRANSPLANTATION

The incidence of diabetes mellitus is growing rapidly, with an estimated 171 million affected individuals in 2000, which is projected to rise to 366 million by 2030[43]. Globally, 50% of all diabetes patients die of cardiovascular diseases and 10%-20% die due to causes related to end-stage renal failure. Diabetes is also a leading cause of renal failure, both in developed and developing countries[44]. Approximately 10% of all diabetes mellitus patients suffer from type 1 diabetes, which is an autoimmune disease characterized by the selective destruction of insulin producing ß-cells in the islets of Langerhans. The onset typically occurs before adulthood, and several factors (both genetic and environmental) initiate or trigger the poorly understood process of autoimmunization that leads to the progressive destruction of ß-cells.

The incidence of childhood-onset type 1 diabetes is rising worldwide, with an overall annual increase of 3.4% for the period between 1995 and 1999[45]. Current estimates for Europe indicate that, from 2005 to 2020, the prevalence of type 1 diabetes in children below the age of 15 will increase by 70%[46]. The demand and need to find a more effective therapy and, if possible, a curative treatment is rising.

The most frequently used therapy for controlling blood glucose involves the exogenous administration of insulin, either by subcutaneous injection or glucose level sensing pumps.

Unfortunately, in a substantial proportion of patients, the inadequate control of blood glucose levels results in secondary microvascular complications, such as retinopathy[47], neuropathy, and nephropathy, as well as macro-vascular disease[48]. When diabetic nephropathy with overt proteinuria has developed, it cannot be reversed, and eventually renal replacement therapy is necessary. However, kidney transplantation alone has no effect on the progression of extra-renal complications. Restoring endogenous insulin production by replacing the insulin-producing ß-cell population would provide a more effective means to halt or even reverse the debilitating complications of diabetes mellitus. Therefore, simultaneous pancreas-kidney transplantation is currently the preferred treatment option for diabetic patients with or approaching end-stage renal failure. Preferably, intervention would take place prior to the development of secondary complications; restoring the ß-cell population by transplanting isolated islets of Langerhans provides an attractive option for the future. In the current field of ß-cell replacement therapy, whole pancreas transplantation is a well-established cure for type 1 diabetes, while islet transplantation is still experimental and only available in a few centers.

1) Whole pancreas transplantation is currently the only approved strategy and has high success rates. Pancreas transplantation constitutes a major surgical intervention, most often performed simultaneously with a kidney transplantation, which limits its acceptability and use to patients with (or approaching) end-stage renal failure.
2) Islets of Langerhans transplantation holds promise but is currently only employed in research settings. Islet transplantation offers the attractive option of minimal invasiveness and, with further improvement in immunosuppressive therapies, early intervention. However, islet transplantation still suffers from poorer results in terms of freedom from exogenous insulin and graft survival rates compared with a whole pancreatic transplant.

This thesis focuses on acute (antibody-mediated) rejection in kidney, simultaneous pancreas-kidney and islets of Langerhans transplantation. One important difference in kidney transplantation alone compared with both whole pancreas and islets of Langerhans transplantation is the fact that kidneys are allocated based on algorithms that include matching criteria for HLA antigens. Simultaneous pancreas-kidney and islets of Langerhans recipients are transplanted if ABO-compatible but without prospective HLA-matching.

2.1 Pancreas transplantation

The discovery by Joseph von Mering and Oscar Minkowski in 1889 that the pancreas is involved in the development of diabetes established a profound research interest in pancreas transplantation. The first successful human simultaneous pancreas-kidney transplantation (SPKT) was performed in 1966 at the University of Minnesota. Although the results of SPKT were initially poor, it was established that insulin independence could be achieved through this procedure. Until 1990, pancreas transplantation was considered an experimental procedure. Since 2000, the American Diabetes Association has adopted pancreas transplantation as an acceptable therapeutic option for patients with end-stage renal failure (ESRF) due to diabetes mellitus on the condition that these

patients also previously received or will receive a kidney transplant. In Europe, pancreas transplantation is an accepted therapeutic modality for patients with type 1 diabetes. In recent decades, further refinement of surgical techniques, better immunosuppressive drugs and regimens, and advancements in patient management have decreased the technical and immunological failure rates of pancreas transplantation and have established the current success rates[49]. As of 2010, over 35,000 pancreas transplants have been reported worldwide to the International Pancreas Transplant Registry (IPTR)[50].

Types of pancreas transplantation

A pancreas allograft is most often transplanted simultaneously with a kidney allograft (SPKT) to restore renal function and cure the underlying disease, type 1 diabetes. SPKT is documented to have a positive effect on the quality of life of recipients[51;52] and may even halt or reverse secondary diabetic complications[53-55]. In SPKT, the pancreas and kidney are procured from one deceased donor. In the period 2004-2008, 73% of all pancreas transplantations in the United States were in the form of an SPKT[56].

In addition to an SPKT, the pancreas can be transplanted alone (PTA) or after a previous kidney transplantation (PAK). Pancreas transplantation alone, or a solitary pancreas, is only performed in patients who suffer from labile diabetes with recurrent severe hypoglycemic episodes with loss of consciousness and/or requiring necessary assistance from a third party[48]. Pancreas after kidney transplantation (PAK) is performed in patients who have previously undergone kidney transplantation (for example, from a live donor) and already receive maintenance immunosuppressive therapy. Another reason for PAK is when the pancreas is lost due to thrombosis or acute rejection after an SPKT, and a repeat pancreas transplantation is performed to ensure glycemic control.

Patient and graft survival after pancreas transplantation

Clinically, pancreas graft success is defined as the freedom from exogenous insulin therapy. In the first year after transplantation, small differences in pancreas graft survival have been found for the three types of transplantation (SPKT, PAK and PTA)[57]. In the long run, however, the results after an SPKT are superior to those after a PAK or a PTA. Pancreas graft survival rates for SPKT recipients were 86% at 1 year and 53% after a 10-year follow-up, while PAK graft survival rates were lower, with a 1-year survival rate of 77% and a 10-year survival rate of 35%. PTA recipients had even poorer graft survival rates with 81% at 1 year and only 26% after 10 years[57]. Patient survival rates after pancreas transplantation have markedly improved over the years, with over 90% of patients still alive 3 years after transplantation for the period between 2004-2008[58].

In two studies that assessed mortality risk following transplantation, SPKT patients were shown to have a lower mortality risk than diabetic patients who were on the waiting list[59;60]. However, the results for the PAK and PTA procedures in these analyses were inconclusive. One study showed an increased mortality risk for transplanted patients compared with conventional therapy for patients on the waiting list[60], while another study reported no difference in survival[59]. These differences may be explained, at least in part, by the different

follow-up periods in these studies. In the first year after pancreas transplantation, there is a relative high perioperative mortality rate, but after this initial period, mortality is low. For the pancreas transplant waiting list, on the other hand, the mortality rate is low in the first year, but increases significantly with time spend on the waiting list.

Finally, various studies found that the quality of life after transplantation is improved[51;52], although the assessment tools are subject to debate. In addition, pancreas transplantation may slow down or even reverse the progression of otherwise progressive and often debilitating complications, such as (recurrent) nephropathy[54], retinopathy[55], neuropathy[53] and macro-vasculopathy[61].

Pancreas transplantation and complications

Although proven to be very successful, SPKT remains a major surgical intervention in a vulnerable patient population with often considerable co-morbidity. Compared to a kidney allograft, the pancreas has relatively low blood flow, which gives rise to specific complications more frequently associated with pancreas transplantation. Transplantation of the pancreas may lead to thrombosis, pancreatitis due to ischemia/reperfusion injury, and/or acute rejection of the graft and is associated with morbidity due to the need for exocrine drainage, either enteric or via the bladder.

Risks, complications, and adverse events related to pancreas transplantation can be divided into short-term procedure-related risks and long-term risks, which are mostly associated with the life-long need for immunosuppressive therapy. Technical failure within the first 3 months is still the most frequent cause of graft loss, followed by patient death with a functioning graft[58]. The causes for early pancreas graft loss due to technical failure are various, with graft thrombosis the most frequent and infection, pancreatitis, anastomotic leakage and bleeding the less frequent causes[56;62]. Drainage of the pancreatic enzymes can be established via the bladder or by enteric anastomosis. Primary enteric drainage has been associated with higher early technical failure rates[58].

After the direct perioperative period, which has a relatively high rate of technical failure, the pancreas graft is potentially exposed to the same threats as the simultaneously transplanted kidney, including threats such as acute rejection, toxicity of immunosuppressive drugs[63], and recurrence of the underlying disease[64]. In the early days of pancreas transplantation, the potential toxicity of steroids to ß-cells became apparent, and today most immunosuppressive regimens are steroid-sparing or avoiding. However, most of the other immunosuppressive drugs that are still widely used are either nephrotoxic or known to hamper ß-cell function[65]. Furthermore, the long-term use of immunosuppressive drugs is associated with an increased risk of developing malignancies, in particular, skin cancers and certain lymphoproliferative disorders[66]. In renal transplantation, malignancies caused by immunosuppressive therapy are the third most common cause of death[67;68]. Use of immunosuppressive agents is also associated with an unfavorable cardiovascular risk profile and an increased risk of cardiovascular events. The increasing number of patients with type 1 diabetes and the current shortage of donor organs available for transplantation pose the

biggest threats to patients with diabetes and end-stage renal failure who are currently on the waiting list for kidney and/or pancreas transplantations.

2.2 Islets of Langerhans transplantation

Islets of Langerhans are the endocrine constituents of the otherwise exocrine pancreas. The islets vary in size[69;70] (ranging mostly between 50-300 µm in diameter) and composition[69;71] and are unequally distributed from head to tail[72]. A mere 1-2% of the pancreas is composed of these endocrine islets, while the bulk of the pancreas is composed of exocrine tissue producing digestive enzymes.

Islets of Langerhans are 'mini-organs' composed of several hormone producing cells, and, to a lesser extent, stromal cells, endothelial cells from the microvasculature, nerves, and immune cells such as dendritic cells (DC). The most abundant cell type of the islet is the ß-cell, which produces insulin and amylin. Islets consist of α-cells producing glucagon, δ-cells secreting somatostatin, PP-cells secreting pancreatic polypeptide, and γ-cells producing ghrelin. The ß- and α-cells maintain blood glucose homeostasis. In type 1 diabetes, the capacity of the ß-cell to produce insulin is destroyed as autoimmune antibodies evoke the selective destruction of these cells. To cure type 1 diabetes, grafting of only the insulin-producing ß-cell mass would, in theory, suffice.

In 1893, 29 years before the discovery of insulin, the use of freshly slaughtered sheep pancreas pieces was explored to cure a human diabetes patient. The 15-year-old recipient died in coma after 3 days[73]. In 1972, the effectiveness of islet transplantation as a therapy to temporarily resolve diabetes was established in a rodent model[74]. The first actual successful human pancreatic allogeneic islet transplantation was performed in 1978[75]. Unfortunately, the extraction of sufficient numbers of functional islets from a human donor pancreas proved to be an obstacle, thereby slowing down these first initiatives.

In the United States, the Food and Drug Administration (FDA) has put allogeneic pancreatic islets under legislation as a drug product and as a biological product. Legislation mandates that, for the isolation of islets as a biologic product, facilities procuring and handling pancreatic islets must adhere to Good Manufacturing Practices. Currently, the use of pancreatic islets for the treatment of type 1 diabetes is considered experimental. To date, approximately 1000 islet transplantations worldwide have been performed, primarily due to the lack of donor pancreata and the still disappointing success rates. Several causes have been proposed for the poor success rates, such as allograft rejection, the instant blood-mediated inflammatory reaction (IBMIR)[76], the recurrence of auto-immune type 1 diabetes[77-79], ß-cell senescence and ß-cell cytotoxicity caused by the immunosuppressive agents[65].

In 2000, a breakthrough publication by a research group from Edmonton reported on the islet transplantation results of 7 islet recipients one year post-transplantation[80]. The 7 recipients remained insulin independent for a median time of 11 months. Although this was a small study, its results stirred much enthusiasm in the diabetes community[80]. In Edmonton the islet isolation and transplantation protocol was changed by improving the islet isolation procedures, by setting an adequate target islet mass for transplantation

including repeated islet implantations and by altering the immunosuppressive regimen. The success of the islet isolation and transplantation protocol developed in Edmonton was confirmed by an international study group in 2006[81]. After the initial improvements by the Edmonton research group, several modifications to the protocol were tested and implemented to further advance the success rate of islet transplantation[82;83].

Given that islet transplantation has not proven as successful as initially anticipated, research is currently aimed at various improvement possibilities. Given that the interactions between the human immune system and human islets of Langerhans after transplantation are difficult if not impossible to monitor and study[84-86], most research is conducted on animal models. Both rat and mouse models are studied to improve isolation conditions and the transplantation process and study the interactions between recipient and host tissue. There is one major discrepancy between animal models and the human transplant setting. In animal models, the islets are most often transplanted underneath the kidney capsule, for numerous practical reasons[82;87;88], as opposed to intra-portal infusion and subsequent liver engraftment of islets in humans.

Patient and graft survival after islet transplantation

Initially, success of islet transplantation was defined as having prolonged insulin independent glucose homeostasis for all treated patients. However, after the first few islet transplantations, it became apparent that insulin independence is difficult to establish. Most patients had to resume exogenous insulin therapy eventually, although insulin requirements to achieve euglycemia were reduced compared with the need before islet transplantation. In these patients, residual C-peptide levels could be detected in the serum of the recipient, which were absent before transplantation, indicating that some transplanted islets still functioned. C-peptide is secreted from the ß-cell together with insulin in a 1:1 molar ratio and, as such, can be used as a surrogate marker for ß-cell function. These residual C-peptide levels are known to reduce the number of hypoglycemic episodes in these patients[89]. Therefore, the reduction of insulin use, the frequency of hypoglycemic episodes, and quality of life[90] can be measured as secondary definitions of islet transplantation success. In 2003, the US FDA Biological Response Modifier Advisory Committee met to discuss an alternative definition of success, which they defined as follows: the restoration of sustained euglycemia (i.e., the absence of hyper-, and hypoglycemia) with no or reduced exogenous insulin requirement[91].

From the patient's perspective, an improvement in quality of life can still represent a successful transplantation, even if insulin dependency recurs. Autocrine insulin production, indicated by blood C-peptide levels, was still detectable even though additional exogenous insulin therapy was required. These residual C-peptide levels were related to improved glycemic control and resulted in reduced patient fear of hypoglycemia[92]. In addition, the health-related quality of life of the diabetes patient was found to be improved after the islet transplantation[93].

Islet transplantation alone (ITA) (i.e., in the absence of renal transplantation) was systematically reviewed by Guo *et al* for the Canadian Institute of Health Economics[94]. As

reported in the 11 key studies included in this systematic review, 30%-69% of the patients were insulin independent 1 year after islet transplantation. Two years after islet transplantation, only 14%-33% of the patients remained insulin independent[94]. The procedure itself has a steep learning curve, and therefore higher success rates are observed in more experienced centers. In a 5-year follow-up study conducted by the experienced Edmonton group between 1999-2004 that included 65 patients, insulin independence rates were reported at ~69% after 1 year, ~37% after 2 years, and 7.5% 5 years after the transplantation. After 5-years of follow-up, C-peptide levels could still be detected in ~80% of the patients[89].

Few data exist on patient survival after islets of Langerhans transplantation because only a limited number of patients have received a transplant to date. Of the 325 adult recipients reported to the Collaborative Islet Transplant Registry, 7 died of causes that had an unknown relationship with the procedure[95]. No randomized, controlled trials have addressed the mortality risk by comparing islet transplantation with the best medical practice. We can speculate, based on the results from pancreas transplantation studies, that the mortality risk will ultimately be reduced for patients who receive a successful islet transplantation[59;60].

Types of islet transplantation, risks, and complications

Similar to the pancreas transplant setting, islets of Langerhans can be transplanted in three different combinations. ITA is the most commonly used procedure. The benefits of this procedure include the possibility of early intervention without major surgical risks, a direct improvement in quality of life, while secondary complications of diabetes can be prevented[90]. However, whether the discontinuation or reduction in exogenous insulin therapy and the improved quality of life outweigh the daily and life-long burden of requiring immunosuppressive drugs and dealing with their side-effects must be carefully determined for each eligible recipient individually[96].

Islet after kidney transplantation (IAK) is another combination in which islets of Langerhans can be transplanted. In this case, the secondary diabetic complications have already evolved and the patient has received a kidney transplant. These patients already receive maintenance immunosuppressive agents, and the clinical decision to proceed with islet transplantation has a different risk-benefit ratio[97].

Every operation or procedure has its potential and specific complications. For islet transplantation there is little chance that a detrimental complication will develop because, unlike whole pancreas transplantation, islet transplantation is a minimally invasive procedure[98]. The main reported infusion-related complications are hepatic bleeding, portal vein thrombosis, and transient elevation of liver enzymes[99]. During the peritransplantation period, the direct contact of islets with blood components in the hepatic portal system is thought to cause an immediate blood-mediated inflammatory reaction (IBMIR)[76], which results in a marginal and inadequate islet mass that actually reaches the liver tissue[100;101]. Post-transplantation, revascularisation[84], rejection, glucose toxicity[102], and immunosuppressive drug toxicity[65;81;94;103] are additional factors that are considered responsible for the further decline of islet allograft survival.

3. SCOPE OF THIS THESIS

The aim of the work described in this thesis was to obtain greater insight into the mechanisms of acute rejection in renal, simultaneous pancreas-kidney, and islets of Langerhans transplantation. Within the scope of acute rejection, the main focus of this thesis was on antibody-mediated rejection (AMR), which was first described in the renal transplant setting, but the pathological process is not completely understood. With the recognition of previously unknown manifestations, such as C4d-negative AMR and focal C4d-positive staining with DSA, the field has become even more complex. Furthermore, in other organ transplant settings, such as the pancreas, even basic evidence of the existence or relevance of AMR is lacking. In this thesis, the role of C4d was examined in the renal transplant setting, in which prospective HLA-matching is the standard of care, before venturing into AMR in the non-HLA matched transplants, which include simultaneous pancreas and kidney and islets of Langerhans transplantation.

Positive C4d staining in the capillaries of transplanted tissue is associated with the presence of donor-specific antibodies in the circulation. In Chapter 2, the long-term outcome of C4d staining in renal allograft biopsies with an acute rejection was studied. The study described in Chapter 2 shows that positive staining for C4d was rare in this cohort of kidney transplant recipients and that the mere presence of a positive C4d staining had no effect on patient and/or graft survival nor could it predict steroid resistance as defined by the need for anti-thymocyte globulin therapy.

To elucidate the role of AMR in pancreas graft failure, a retrospective study of pancreas biopsies was conducted in collaboration with the University of Maryland. The study presented in Chapter 3 shows that AMR does occur after pancreas transplantation, requires the presence of both diffuse interacinar C4d staining and DSA for its diagnosis, and is associated with poorer graft survival.

In the study described in Chapter 4, pancreas graft transplantectomies performed within one year after the SPKT were compared with pancreas grafts that functioned at least 4 years after the SPKT. This study shows that early pancreas graft failure was associated with older donor age (>45 years of age) and female gender of the recipient. Furthermore, AMR (defined by the C4d-positive interacinar capillary staining in the presence of DSA and morphological evidence of graft injury) was present in 7 out of 33 cases of early pancreas graft loss.

In Chapters 5 and 6, we reviewed islets of Langerhans transplantation. Chapter 5 is a clinical review of the status of human islet transplantation. Structural and morphological changes of islets transplanted in rodents were assessed in Chapter 6, focusing on blood vessel revascularization, neurogenesis and lymphatic revascularization.

Cellular immune modulation in islet transplantation is the topic of Chapter 7. Using donor-derived tolerogenic DC, we aimed to prevent rejection in a fully MHC-mismatched rodent model. The study described in Chapter 7 showed that the use of these tolerogenic DC provoked an antibody-dependent accelerated destruction of the allograft.

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2

C4D STAINING IN RENAL ALLOGRAFT BIOPSIES WITH EARLY ACUTE REJECTION AND SUBSEQUENT CLINICAL OUTCOME

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Clinical Journal of the American Society of Nephrology 2011; 6: 1207-1213

ABSTRACT

Background and objectives

Diffuse C4d staining in peritubular capillaries (PTCs) during an acute rejection episode (ARE) is the footprint of antibody-mediated rejection. In current clinical practice, diffuse C4d+ staining during acute rejection is regarded as an inferior prognostic sign. This case-control study investigated the prognostic role of mere C4d staining for graft outcome during an ARE in a well defined cohort of similarly ARE-treated patients.

Design, setting, participants, & measurements

All kidney transplant recipients in the authors' center from January 1, 1995 to December 31, 2005 were reviewed. From these patients, 151 had a clinical ARE. Paraffin and/or frozen material was available for 128 patients showing a histologically proven ARE within the first 6 months after transplantation. All ARE patients were treated similarly with high-dose pulse steroids and in the case of steroid unresponsiveness with anti-thymocyte globulin. Biopsies were scored according to Banff criteria. Frozen and paraffin sections were stained by immunofluorescence (IF) and immunohistochemistry (IHC) for C4d, respectively, and scored for PTC positivity.

Results

Diffuse C4d+ staining in PTCs was found in 12.5% and 4.2% sections stained by IF or by IHC, respectively. Four patients showed diffuse positive staining with both methods but showed no different risk profile from other patients. No relation between C4d staining and clinical parameters at baseline was found. C4d staining was not associated with steroid responsiveness, graft, or patient survival.

Conclusions

This study shows that C4d staining is not related to clinical outcome in this cohort of histologically proven early AREs.

INTRODUCTION

In renal transplantation, long-term graft survival strongly depends on events occurring early (i.e., within 1 year) after transplantation. A major event is the occurrence of an acute rejection episode (ARE), which is a main risk factor for the development of graft loss over time[1-3]. An ARE can be mediated by cellular- and antibody-mediated reactions. In antibody-mediated rejection (AMR), donor-specific antibodies (DSAs) can bind complement factors and initiate the classical pathway of complement activation. During the activation cascade, C4d, a complement split product, is formed. C4d has the capacity to covalently bind to target molecules on the endothelium of peritubular capillaries (PTCs) and is therefore regarded as a footprint of AMR[4]. Sensitivity (95%) and specificity (96%) of diffuse C4d staining in PTCs for the presence of DSAs is high[5]. Other studies have found a strong relation between diffuse C4d staining of PTCs and the presence of DSAs[6-8].

Acute AMR is associated with nonresponsiveness to standard rejection therapy of steroids (i.e., steroid resistance) and has a detrimental effect on graft outcome[8-12]. The outcome of AMR significantly improves when promptly treated with aggressive immunosuppressive regimens[13 17]. The combination of circulating DSAs, histomorphological features of AMR, and diffuse C4d deposition is currently the gold standard in the diagnosis of AMR[16]. Previous studies found unfavorable graft outcome for C4d+ stained biopsies[7;18-20]. This might suggest that sole C4d deposition could be used as prognostic marker and also guide more aggressive therapy.

In 1993, Feucht et al. were the first to describe C4d staining in renal transplant biopsies[21]. In 93 renal allografts showing dysfunction after transplantation, an incidence of 46.2% diffuse C4d and 8.6% focal C4d staining was found. C4d staining significantly correlated to 1-year graft survivals of 57%, 63%, and 90% in diffuse, focal, and negative staining, respectively. Subsequent studies showed an unfavorable graft out-come in diffuse C4d+ stained biopsies taken on clinical indication[6-8;18-22]. However, these studies have five major drawbacks. First, most studies included biopsies with a broad range of histologic diagnoses, causing heterogeneity. Second, follow-up time (average 5 years) was relatively short in most studies. Third, in some studies rejection therapy differed between C4d+ and C4d- patients. Fourth, some studies used other criteria to determine C4d positivity than the Banff criteria. Fifth, some studies included more than one biopsy per patient and used the biopsy that stained most positive for C4d as the index biopsy.

The prognostic value for graft survival of untreated C4d+ AREs has not been investigated in a cohort of patients with AREs who were not differently treated for this. We questioned whether C4d+ AREs show a difference in long-term renal function compared with C4d- AREs when treated according to the same therapy regimen.

MATERIALS AND METHODS

Patients

We reviewed all 723 patients who received a renal transplant in our center from 1995 until 2006, of which 498 (68.9%) never had a rejection episode. One hundred and twenty-eight patients who had a clinically suspect and histologically proven first ARE within 6 months after transplantation (17.7% of all single renal transplant patients) were included in this study. A total of 104 frozen and 118 paraffin-embedded renal biopsies were available, with an overlap of 94 patients. Maintenance and rejection therapy were analyzed.

All patients received calcineurin inhibitor (CNI)-based maintenance immunosuppression (Neoral: cyclosporine [CsA] microemulsion [86%]; or Prograft: tacrolimus [Tac] [14%]) and corticosteroids (P) with or without mycophenolate mofetil (MMF; 62.5%). Since 2000, all patients received prophylactic therapy with an IL-2 receptor antagonist (basiliximab), and one patient received induction with anti-thymocyte globulin (ATG). Of the 128 patients with biopsy-proven acute rejection, 30% received prophylactic antibody therapy.

AREs were treated with high-dose methylprednisolone (1 g intravenously for 3 consecutive days). If serum creatinine (SCr) did not return to baseline within a 20% range, a 10-day course of ATG at a dose 5 mg/kg was given. Steroid resistance was defined as the use of ATG therapy. Biopsies were taken before steroid therapy was started. Patients who had undergone a pancreas-kidney or other combined organ transplantation were excluded from this study. All patients had a negative complement-dependent cytotoxicity crossmatch before transplantation.

Clinical data

Donor and recipient age and sex, donor source, number of rejection episodes and re-transplantation, percentage panel reactive antibodies (PRAs) present before transplantation, time between transplantation and the occurrence of the ARE, HLA mismatches, delayed graft function, patient and graft survival, quantitative proteinuria, and steroid resistance were analyzed. Graft failure was defined as return to dialysis. Graft failure was censored for patient death.

SCr was used as a surrogate marker for renal function over time. During patient follow-up, SCr was measured at regular check-up times and on additional clinical indications. SCr at 1 year after transplantation was taken as baseline. We used time until patients reached a SCr value of 150% and 200% of their SCr at 1 year as an indicator of renal function follow-up. In a Kaplan–Meier survival analysis, time to these events was compared between the C4d+ and C4d- group.

Histology

For routine diagnostic evaluation, paraffin-embedded sections were cut and stained with silver methamine, hematoxylin and eosin, and periodic acid–Schiff. Two independent pathologists (I.B. and N.G.) blindly scored biopsies for morphologic features by light microscopy using the Banff classification[16].

C4d staining

Staining protocols have been described previously[23-25]. Immunofluorescent staining was performed on 4-µm frozen sections with monoclonal mouse anti-human C4d antibody (Quidel, San Diego, CA). Immunohistochemical staining was performed on 4-µm paraffin sections with polyclonal rabbit anti-human C4d antibody (Biomedica Gruppe, Wien, Austria). Two independent and blinded observers (IF: M.E. and K.K.; IHC: I.B. and M.vG) semiquantitatively scored all sections. C4d staining in PTCs was evaluated according to the area percentage of positive staining in the renal cortex as described in the Banff '07 criteria[16]. Three C4d staining groups were made: negative (<10% of PTCs), focal (10% to 50% of PTCs), and diffuse positive (>50% of PTCs) staining. Necrotic and fibrotic areas (if present) were excluded from evaluation.

Statistical analyses

SPSS version 16.0 was used to perform statistical analyses. For continuous variables, means with standard errors were calculated and differences were assessed by independent sample t tests. For categorical data, crosstabs were made and differences were calculated by Fisher exact test. Univariate analyses using a logistic regression model were performed, and a multivariate analysis was performed using a logistic regression model. Survival analyses were performed using Kaplan–Meier survival curves and logrank tests to test for differences between C4d groups. The significance threshold was set at $p < 0.05$.

RESULTS

Patient characteristics

Of the 128 patients included in this study, 77% received a donor kidney from a deceased donor and 23% received a kidney from a living donor. Mean follow-up time was 7.3 years (±3.9).

Immunosuppressive maintenance therapy was comparable for all patients in the cohort, consisting mostly of a CNI (CyA or Tac) in combination with low-dose P alone or P with IL-2 receptor or MMF.

Prevalence of C4d positivity

We investigated C4d staining patterns by IF (frozen sections) and IHC (paraffin-embedded sections) staining techniques in the same cohort and related these with clinical outcome. Thirteen of the 104 (12.5%) sections stained by IF and 5 of the 118 (4.2%) sections stained by IHC showed diffuse C4d+ staining (Table 1). Focal C4d+ staining was seen in six (5.8%) and two (1.7%) of the patients, respectively.

Four patients showed diffuse C4d+ staining with both techniques (Table 2). Overall, these patients were not remarkably different compared with the whole cohort in age, maintenance therapy, donor age and sex, PRAs, delayed graft function, HLA mismatches, rejection episodes, timing of rejection, steroid resistance, or vascular rejection. One of these patients lost graft function 10 days after transplantation and transplantectomy was

Table 1 | Prevalence of C4d staining categories of PTC stained by IF or IHC.

C4d staining category	Prevalence IF	Prevalence IHC
Negative	85 (81.7%)	111 (94.1%)
Focal positive	6 (5.8%)	2 (1.7%)
Diffuse positive	13 (12.5%)	5 (4.2%)
Total	104 (100%)	118 (100%)

PTC peritubular capillaries; IF immunofluorescence on frozen tissue; IHC immuno-histochemistry on paraffin-embedded tissue. Data presented as n (%).

performed. The explanted organ showed severe diffuse vascular rejection with necrosis and graft ischemia. The other three patients maintained stable graft function and their grafts survived during the follow-up period (range 1.86 to 6.43 years).

As depicted in Table 2, one biopsy was diffusely C4d+ stained by IHC, but no IF staining was performed because of a lack of frozen material. Seven diffusely C4d+ biopsies with IF staining were negative using IHC. No paraffin material was available for IHC staining of two diffusely C4d+ biopsies using IF. Of the six focal C4d+ cases using IF, only one was focal C4d+ using IHC; all others were negative. All biopsies negative for C4d staining by IF were also negative in the IHC staining.

Statistical analyses were performed on IF- and IHC-stained cases. No differences were found in the IHC-stained cases. The results hereafter are only those analyzed using the IF staining. Diffuse C4d staining was called "C4d+" and focal and negative C4d staining were combined as one group, called "C4d". Others categorized C4d staining in the same way[5].

Table 2 | Overlap between C4d staining categories using IF and IHC.

C4d IF→ C4d IHC↓	Negative	Focal Positive	Diffuse Positive	Total
Negative	77	5	7	89
Focal Positive	0	1	0	1
Diffuse Positive	0	0	4	4
Total	77	6	11	94

IF immunofluorescence on frozen tissue; IHC immunohistochemistry on paraffin-embedded tissue.

C4d and Banff histology

C4d staining (IF) only related to the absence of tubular atrophy (8.3% versus 50.6%; p = 0.006) and not to any of the other individual components of the Banff score (Table 3). After IF and IHC C4d staining and blind scoring of biopsies, the biopsies of 14 patients showing diffuse C4d+ were subsequently reassessed (I.B.) to investigate histomorphological

characteristics (e.g., granulocytic infiltrate, microthrombi, peritubular capillaritis [Table 3], or necrotizing vascular rejection) indicative of a possible AMR[4]. None of those biopsies showed characteristics indicative of an antibody-mediated component.

Table 3 | Banff characteristics in relation to IF C4d staining patterns.

Banff characteristic	C4d+ (*n*=12) **number (%)**	C4d– (*n*=85) **number (%)**	**P**
Glomerulitis	6 (50)	29 (34.1)	0.34
Chronic glomerular changes	1 (8.3)	5 (5.9)	0.56
Tubulitis	7 (58.3)	49 (57.6)	1.00
Tubular atrophy	1 (8.3)	43 (50.6)	0.006
Interstitial infiltrate	5 (41.7)	48 (56.5)	0.37
Interstitial fibrosis	2 (16.7)	38 (44.7)	0.12
Intima arteritis (vascular rejection)	4 (33.3)	33 (43.4)	0.75
Chronic vascular changes	5 (41.7)	41 (52.6)	0.55

C4d+ diffuse positive stained slides versus C4d- focal positive or negative stained slides. All characteristics were split in presence (score 1-3) and absence (0), except for glomerulitis, tubulitis, interstitial infiltrate, and peritubular capillaritis where we dichotomized for mild (0,1) and severe (2,3). Fisher exact test was used to calculate significances of differences between groups. Values expressed as mean and number (%).

C4d and baseline clinical characteristics

Baseline clinical characteristics are shown in Table 4. Only donor age was significantly lower in the C4d+ group (38 ± 15 years versus 48 ± 14 years; p = 0.02). No significant difference in number of living related donors was observed between the C4d+ and C4d- group (1 of 13 [7.7%] versus 13 of 91 [14.3%], respectively, p = 1.00; data not shown). The interquartile range for follow-up was 6.46 years (3.47 to 10.45) and 6.80 years (3.80 to 10.57) for the C4d+ and C4d- group, respectively.

In a multivariate analysis, C4d was only related to donor age and not to any other clinical parameter (Table 4). Univariate analyses for clinical characteristics were also performed separately for living and deceased donors (data not shown). Results concerning differences in graft outcome between C4d+ and C4d- groups were similar in living and deceased donor recipients (younger donor age in the C4d+ group in the living kidney donors [38 ± 8 years versus 51 ± 12 years; p = 0.049]).

Twelve of the 13 C4d+ patients were primarily treated with high-dose P; one patient primarily received ATG. Eight patients subsequently received ATG because of non-responsiveness to steroids. These numbers were similar in the C4d- group.

C4d and clinical outcome

Incidence of steroid resistance was comparable between the C4d+ and the C4d- group (54%, and 47%, respectively; p = 0.770). Patient survival was similar between the two

Table 4 | Baseline clinical characteristics between C4d+ (diffusely stained on IF) and C4d- (focally positive or negative on IF staining) groups.

			Univariate analyses			Multivariate analyses		
Characteristic	**C4d+**	**C4d-**	**OR**	**95% CI**	**P**	**OR**	**95% CI**	**P**
Sex (m/f)	9/4 (69.2/30.8)	59/32 (64.8/35.2)	1.22	0.35-4.28	0.76	1.03	0.17-6.25	0.98
Age (yr)	50.0 ± 11.4	46.5 ±12.5	1.02	0.98-1.08	0.34	1.04	0.96-1.12	0.37
Donor sex (m/f)	2/11 (15.4/84.6)	36/54 (40.0/60.0)	0.27	0.06-1.30	0.10	0.24	0.03-1.71	0.16
Donor age (yr)	37.9 ± 14.9	48.0 ±13.7	0.95	0.91-0.99	0.02	0.94	0.89-0.99	0.03
Donor type (living/deceased)	4/9 (30.8/69.2)	20/71 (22/78)	1.58	0.44-5.66	0.48	1.88	0.28-12.56	0.51
HLA-A mm (0/1/2) (%)	31/62/8	36/54/10	1.25	0.35-4.38	0.73	1.49	0.23-3.58	0.67
HLA-B mm (0/1/2) (%)	8/77/15	29/55/16	4.84	0.60-39.2	0.14	0.81	0.04-16.95	0.89
HLA-DR mm (0/1/2) (%)	15/69/15	36/61/4	3.10	0.65-14.9	0.16	3.48	0.30-40.55	0.32
No of transplantation	1.31 ±0.63	1.16 ±0.45	1.66	0.61-4.55	0.32	2.99	0.60-14.83	0.18
PRA pre-transplantation (%)	10.6 ±22.9	6.5 ±16.3	0.56	0.12-2.52	0.45			
Delayed graft function (days)	1 (7.7)	26 (28.6)	0.21	0.03-1.68	0.14	0.21	0.01-3.58	0.28
Induction therapy	3 (23.1)	29 (31.9)	0.60	0.16-2.50	0.52			
Calcineurin inhibitor (Cya/Tac)	12/1 (92.3/7.7)	77/13 (85.6/14.4)	0.50	0.06-4.12	0.52			
MMF	7 (53.8)	58 (63.7)	0.66	0.21-2.14	0.49			
Steroid-resistance	7 (53.8)	43 (47.3)	1.30	0.41-4.18	0.66			
Timing of rejection (days)	25.92 (6-156)	33.42 (3-178)	0.99	0.97-1.01	0.50	1.00	0.98-1.02	0.83
Rejection episodes (no)	1.69 ±0.63	1.86 ±0.97	0.80	0.39-1.65	0.55	1.02	0.45-2.32	0.95
Interstitial Infiltrate	5 (41.7)	48 (56.5)	0.55	0.16-1.88	0.34	0.73	0.12-4.69	0.75
Tubulitis	7 (53.8)	49 (57.6)	1.03	0.30-3.50	0.96	1.08	0.12-9.94	0.95
Vascular rejection	4 (33.3)	33 (43.4)	0.65	0.18-2.35	0.51	0.47	0.09-2.57	0.39

Donor age was significantly different between groups. HLA-DR mismatches seem to be more often present in the C4d+ group, though this does not reach statistical significance. PRA (panel reactive antibody) levels were divided in three groups for analysis, ≤5%, 5-85%, ≥85%.Multivariate analysis outcome. Clinical and histological risk factors were included in a multivariate logistic model. Donor age remained significantly different between C4d+ and C4d- groups. Other characteristics were not significantly different between C4d groups. No risk profile for C4d staining could be established. * $p<0.05$ Note: Values are expressed as mean with a ± standard deviation, as number with percentage in brackets, or as mean with minimum and maximum days in brackets. CyA cyclosporin; mm, mismatches; MMF, mycophenolate mofetil; no, number; Tac, tacrolimus.

C4d staining categories (data not shown). One-year graft survival was 92% for the C4d+ group and 97% for the C4d- group, whereas 5- and 10-year graft survivals were 92% and 74% for the C4d+ group and 92% and 82% for the C4d- group, respectively ($p > 0.05$; Figure 1A). Results concerning differences between the C4d+ and C4d- group were similar between living and deceased donors.

No difference was found between the C4d+ and C4d- group for renal function over time, as measured by the time when SCr reached 150% or 200% of its value at 1 year after transplantation. Both showed a similar curve in the Kaplan–Meier analysis for time and the percentage of events of reaching 150% and 200% SCr values ($p = 0.981$ [Figure 1B] and $p = 0.482$, respectively). No differences were found in development of proteinuria (g/24 h) between the C4d+ and C4d- group.

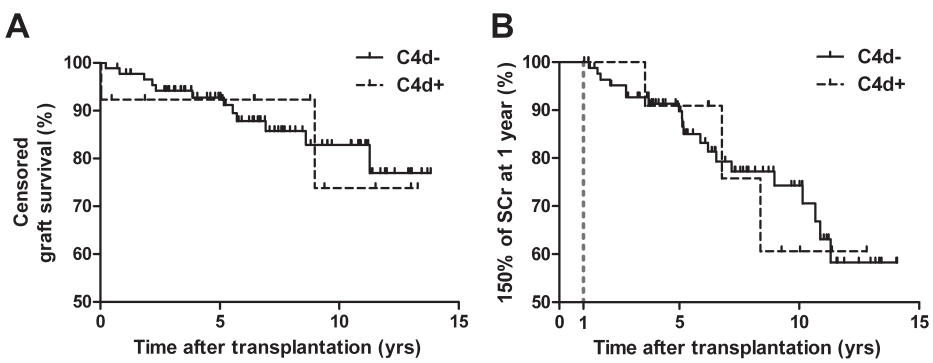

Fig 1 | (A) Graft survival and (B) renal function follow-up for patients with diffuse C4d+ stained PTCs using IF (dotted line) and focal C4d stained/negative C4d stained PTCs using IF (continuous line). (A) No significant difference in graft survival was observed between groups (*P*= 0.969). (B) Renal function follow-up was indicated by a SCr value of 150% of SCr at 1 year posttransplantation. No significant difference in renal function over time was observed between groups (*P*=0.981). Both were tested using Kaplan–Meier analysis.

DISCUSSION

The occurrence of an early ARE is a risk factor for loss of renal allograft function over time. In this study, C4d staining patterns in a group of similarly treated patients during their first ARE were investigated. We compared C4d staining patterns in biopsies of patients with a histologically proven first ARE within 6 months after transplantation. Patients in this retrospective cohort were all treated for their ARE according to the same protocol, firstly with high-dose steroids and in the case of steroid resistance with ATG. Biopsy sections were stained by two techniques: IF on frozen and IHC on paraffin-embedded tissue sections. Diffuse C4d+ staining was seen in 12.5% of IF- and in 4.2% of IHC-stained sections. We found no clinical or histologic risk profile related to C4d+ staining. In addition, we

found no difference in occurrence of steroid resistance or graft survival for patients who in retrospect showed a C4d+ or a C4d- ARE. We conclude that the C4d staining pattern during an early ARE does not predict renal allograft function or survival over time.

Since the introduction of diffuse C4d staining as a marker for antibody-mediated activity in the renal transplant biopsy, several studies concluded that C4d staining of biopsies with allograft dysfunction could be used as an adequate predictive marker for graft outcome[6-8;18;20-22;26]. However, previous studies had several drawbacks, as described in the Introduction section, which could explain the discrepancies between those studies and our study.

The consistent histologic findings of an ARE and the consistent treatment of that episode makes our patient group unique. Graft survival in our cohort of patients was excellent (overall 10-year graft survival: 77.9% ± 4.7%). Our C4d+ ARE biopsies are most likely not indicative for AMR, but perhaps of a more subclinical type. The presence of C4d in biopsies might reflect some antibody being present in a T cell predominant acute rejection, rather than it being an AMR. Aside from tubular atrophy, no concurring histopathological parameters could be found. It is not known why there are significantly more biopsies showing tubular atrophy in the C4d- group. It is possible that the association of C4d- staining with tubular atrophy may reflect the younger age of donors in the C4d+ group. More research in a new patient cohort is required to elucidate this finding. Furthermore, none of the clinical characteristics related to AMR (e.g., recipient being female and/or young, high PRAs, number of successive transplantations, and number of HLA mismatches) were significantly more abundant in the C4d+ group. It is possible that our C4d+ ARE biopsies are of a more subclinical type, which might be due to better HLA matching and adequate DSA screening techniques before transplantation and our donor allocation protocol. We therefore strongly recommend adequate screening of patients pretransplantation and exclusion of any unacceptable mismatches.

Nickeleit et al. found no difference in outcome between C4d+ and C4d- AREs[27]. However, in this study, C4d+ patients were treated more aggressively and the paper concluded that additionally treated C4d+ AREs may hold the same prognosis as C4d- AREs. In the study presented here, we show that patients with a C4d+ ARE who did not receive additional acute rejection treatment have the same graft outcome as a C4d- ARE. This could be because C4d deposition may indicate a state of graft accommodation that does not lead to graft failure[28]. The question remains how C4d as a marker for graft accommodation can be discriminated from C4d deposition due to ongoing AMR. Furthermore, we explicitly studied whether the presence of diffuse C4d+ staining during an ARE was associated with graft survival if not additionally treated. It is not known whether C4d+ patients (or a subgroup of C4d+ patients) would have improved graft function if they would have been more aggressively treated. We therefore do not state that additional therapy in C4d+ ARE patients is not indicated, but we encourage further investigation on this subject.

The incidence of C4d staining in our cohort seems to be rather low compared with earlier studies. In various studies a range of 10% to 55% diffuse C4d+ staining is seen using either staining technique[5-8;18-21;26;27;29]. Different explanations for this can be given.

Inclusion criteria in this study were tightly controlled because we required all patients to have a histologically proven ARE, whereas other studies show heterogeneity with regards to biopsy inclusion criteria, resulting in a broad histomorphological spectrum. Also, the time point at which the biopsies were taken differed between studies. There could be differences in C4d+ incidence between centers, as has also been suggested by Mengel et al.[30]. In a study with protocol biopsies stained for C4d, they reported that C4d incidence was related to the center the biopsy was obtained from. This could be due to differences in laboratory techniques, transplantation procedures, or therapy regimens between centers.

Until now, few studies compared C4d staining techniques with IF and IHC. The C4d staining protocol used to be applied on frozen sections by IF using a monoclonal antibody[21]. Recently, an IHC method for paraffin-embedded sections has been developed using a polyclonal antibody against C4d. Seemayer et al. investigated both staining techniques on inter- and intra-observer variation and found a favorable result for IF-stained frozen sections ($\kappa = 0.9$ [IF] versus $\kappa = 0.3$ [IHC])[31]. However, in that study, C4d staining by both staining techniques was not investigated in relation to graft outcome over time. Also, in the most recent Banff criteria it was noted that C4d staining by IF showed more positivity and is possibly more sensitive, but both C4d staining techniques show strong relations with DSAs and it is still unclear which staining technique is to be preferred[14]. We are the first to investigate C4d staining patterns by both staining techniques in the same cohort and relate it with clinical outcome. We found less C4d positivity in IHC-stained sections compared with IF sections and found no relation to outcome.

There are several drawbacks to our study. First, this was a single-center study; therefore, the results might be difficult to extrapolate to other studies. Furthermore, although this is a relatively large patient cohort, few C4d+ ARE patients were found. In addition, because of the retrospective nature of the study, presence of DSAs at the time of biopsy was not measured. However, earlier studies repeatedly showed that C4d staining was strongly related to DSAs[5;8].

In conclusion, clinicians might feel the urge to more aggressively treat patients with C4d positivity during a histologically proven ARE. However, this retrospective study shows that C4d staining is not related to clinical outcome in this large cohort of first histologically proven early (<6 months) rejection episodes of patients who were not additionally treated.

ACKNOWLEDGMENTS

We gratefully acknowledge Annemieke van der Wal for her excellent technical support. Part of this material was presented at the annual meeting of the American Society of Nephrology, October 27 through November 1, 2009, San Diego, CA; at the annual meeting of the Dutch Transplantation Society, March 26 through 28, 2008, Zeewolde, The Netherlands; and at the annual meeting of the Dutch Association for Pathology, April 22 and 23, 2008, Zeist, The Netherlands.

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3

PANCREAS ALLOGRAFT BIOPSIES WITH POSITIVE C4D STAINING AND ANTI-DONOR ANTIBODIES RELATED TO WORSE OUTCOME FOR PATIENTS

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American Journal of Transplantation 2010; 10: 1669-1676.

ABSTRACT

C4d+ antibody-mediated rejection following pancreas transplantation has not been well characterized. Therefore, we assessed the outcomes of 27 pancreas transplantation patients (28 biopsies), with both C4d staining and donor-specific antibodies (DSA) determined, from a cohort of 257 patients. The median follow-up was 50 (interquartile range [IQR] 8–118) months. Patients were categorized into 3 groups: group 1, patients with minimal or no C4d staining and no DSA (n=13); group 2, patients with either DSA present but no C4d, diffuse C4d+ and no DSA or focal C4d+ and DSA (n=6); group 3, patients with diffuse C4d+ staining and DSA (n = 9). Active septal inflammation, acinar inflammation and acinar cell injury/necrosis were significantly more abundant in group 3 than in group 2 (respective p-values: 0.009; 0.033; 0.025) and in group 1 (respective p-values: 0.034; 0.009; 0.002). The overall uncensored pancreas graft survival rate for groups 1, 2 and 3 were 53.3%, 66.7% and 34.6%, respectively (p=0.044). In conclusion, recipients of pancreas transplants with no C4d or DSA had excellent long-term graft survival in comparison with patients with both C4d+ and DSA present. Hence, C4d should be used as an additional marker in combination with DSA in the evaluation of pancreas transplant biopsies.

INTRODUCTION

Pancreas transplantation is a widely accepted treatment choice for diabetic patients. By December 31, 2003, more than 21000 pancreas transplants had been performed worldwide[1]. The 1-year survival of simultaneous pancreas kidney transplantation (SPKT) has greatly improved over the last decade, with rates of immunological graft failure of only 2% during 1996–2002 in technically successful SPKT. Rejection can occur through the immunological pathways of acute cell-mediated rejection (ACR) or antibody-mediated rejection (AMR).

C4d is a complement degradation product of complement factor C4, which is a footprint of the classical pathway of the complement cascade. C4d can be identified in tissue, and is a reliable marker of AMR in transplanted kidneys, intestines, heart and lungs[2-6]. The typical histopathological and clinical findings in AMR are less well defined in pancreas than in other transplanted organs, and the role of C4d in relation to AMR is only beginning to be described. In a recent study, C4d+ immunolabeling of interacinar capillaries was significantly associated with donor-specific antibodies (DSA) and dysfunction of the pancreas allograft[7].

The diagnostic relevance and therapeutic consequences of positive C4d staining have been investigated extensively in relation to kidney transplantation. For other solid organ transplantations, knowledge of AMR and the role of C4d varies. In small bowel transplants, for instance, the relationship between C4d+ staining and AMR is less pronounced than in the kidney[3]. For pancreas transplantation, which is often performed simultaneously with kidney transplantation, very little is known about the significance of C4d positivity. In the current study, we illustrate the C4d staining patterns in transplanted pancreases, the role of C4d in establishing a diagnosis of AMR, and their relationship with the long-term outcome of graft recipients from the combined experiences of two transplantation centers.

PATIENTS AND METHODS

Patient selection

The electronic records of the Department of Pathology at the Leiden University Medical Center (LUMC) and the Department of Pathology at the University of Maryland Medical Center (UMMC) were searched resulting in the identification of 257 patients with pancreas allograft transplantation; 109 from LUMC (between 1991 and 2001) and 148 from UMMC (between 2000 and 2007). Of the 257 patients, 56 biopsies were available for this study (37 from UMMC and 19 from LUMC), and 27 patients had DSA information. From the LUMC, 26 preoperative pancreas transplant biopsies were available which were used to establish pre-transplantation reference values for C4d staining of pancreas tissue. Clinical information on donor variables (gender and age at time point of death); recipient variables (age at time of transplantation, gender, re-transplantation, pregnancy and transfusion history and need for pre-emptive dialysis); transplantation-related factors (panel-reactive antibodies, human leukocyte antigen (HLA)-A, -B and

-DR mismatches, cold ischemia time and induction therapy); and post-transplantation features (immunosuppressive regimen, acute rejection history, rejection treatment, time and indication for biopsy, cause of allograft loss, cause of death and follow-up in months of the pancreas allografts) were obtained from UMMC and LUMC medical records after approval for the study by the respective Institutional Review Boards.

Biopsies

All pancreas transplant biopsies were performed because of clinically suspected graft rejection, except for 1 protocol biopsy (Table 1). From all biopsies, 4-µm sections were cut and stained with hematoxylin and eosin (H&E).

Immunohistochemical C4d staining

At LUMC, C4d immunohistochemical staining was performed on freshly cut formalin-fixed 4-µm paraffin sections of pancreas. Sections were deparaffinized, antigen was retrieved with TRIS/EDTA, pH 9.0, and the sections were incubated with a 1:25 dilution of rabbit anti-C4d polyclonal antibody (MP products, Biomedica, Austria) for 30 min. Subsequently, endogenous peroxidase was blocked and the EnVision™ Detection Systems Peroxidase/DAB, Rabbit/Mouse (DAKO cytomation, Glostrup, Denmark) was used for development.

At UMMC, C4d immunostaining was performed on 5-µm formalin-fixed paraffin sections using an automated immunohistochemical stainer (Ventana Medical Systems, Tucson, AZ) and labeled with a 1:50 dilution of anti-C4d rabbit polyclonal antibody (American Research Products, Inc., Belmont, MA). Target detection was performed using an indirect biotin-avidin system incorporating diaminobenzidine. Endogenous peroxidase quenching and biotin blocking were performed on-instrument with kit reagents (Ventana Medical Systems, Tucson, AZ). All stained sections were counterstained with hematoxylin for orientation.

Grading

The H&E-stained sections from the pancreas biopsies were scored according to the Banff working proposal of 2008[8]. Positive C4d staining patterns in interacinar capillaries were scored (Figure 1C). Diffuse C4d positivity was defined as >50% positively staining interacinar capillaries, focal C4d positivity was 5% to 50% positively staining interacinar capillaries, and minimal positive staining was <5% positively staining interacinar capillaries or a negative staining pattern. Table 2 lists the scorings of all individual parameters in relation to graft outcome.

Immunosuppressive therapy

At LUMC, standard maintenance therapy consisted of prednisone (P), cyclosporine (CsA) and azathioprine until May 1995. Transplant recipients after May 1995 received P, CsA and mycophenolate mofetil (MMF). Three patients received a 10-day course with 5 mg/day OKT3 as induction therapy. Acute rejection episodes were treated according to a standard protocol consisting of methylprednisolone (MP) 1 g intravenously for 3 consecutive days for the first rejection episode; a 10-day course of antithymocyte globulin (ATG) at a dosage

of 5 mg/kg guided by absolute lymphocyte counts at the second rejection episode and again MP for the third rejection episode. None of the patients were treated for AMR.

Immunosuppressive protocol at UMMC included induction therapy with ATG (rabbit) (years 2000–2006) dose of 7–10 mg/kg or alemtuzumab (Alm) (2006–current) 30 mg. Steroid bolus of P 500 mg was administered intraoperatively and tapered to either low dose (before 2004) or off over 21 days (after 2004). Maintenance therapy included tacrolimus and MMF. Rejection therapy was tailored based on clinical suspicion, C4d staining and presence of detected antibody; and could include additional steroid therapy, ATG, plasmapheresis (PP) and intravenous immunoglobulin (IVIg).

DSA assessment

At LUMC, patient serum samples from 14 of 19 biopsies from 18 patients (1 patient had 2 biopsies) 1 month post-transplantation were procured from –80°C storage. A LAT™ mixed class I & II ELISA (One Lambda, Canoga Park, CA) was carried out to detect the presence of HLA class I and class II antibodies. The ELISA was conducted according to protocol with OD readouts at 630 nm. Afterward, seven positive patients were assessed for the specificity of the antibodies with a complement-dependent cytotoxicity (CDC) test.

At UMMC, DSA were identified in the patient serum samples at the time of biopsy using the Luminex 100 IS System (Luminex, Austin, TX). High definition single beads were used with a mean intensity of fluorescence (MFI) cutoff value for one single antibody at MFI 1000. When three antibodies were present, for example, the MFI cutoff was 3000.

Statistical analyses

Raw data were processed using descriptive statistics and graphical representations. For uncensored graft survival, graft failure was defined as resuming insulin therapy or patient death with functioning graft. For censored graft survival, graft failure was defined as resuming insulin therapy; patient death with functioning graft was considered as lost to follow-up. Kaplan–Meier survival curves were compared with the log-rank test for uncensored and censored graft loss. Significance for all tests was set at 0.05. In Table 2 and Figure 2 the differences between groups, C4d–/C4d+ and DSA negative or positive were assessed by means of the chi-squared test, with * $p<0.05$ and ** $p<0.001$. Statistical calculations were performed using SPSS 16.0 for Windows (SPSS, Chicago, IL).

RESULTS

Patient demographics

The mean age of the recipients was 46 ± 9 years, 45% were female and 91% were Caucasian. All the recipients had diabetes and 48% had hypertension as co-morbidity. The mean HLA mismatch was 4.4 ± 1.4. The median follow-up was 50 (IQR 8–118) months. Table 1 gives an overview of patient and donor demographics, clinical parameters, biopsy indication and treatment in relation to pancreas allograft function. No significant

Patient No.	G	Age at Tx	sex	Re-Tx	Preg-nancy	Donor sex, age	PRA (%)	Transfusion till biopsy	Pre-emptive	CIT (hour)	Tx type	HLA mm	Induction therapy	Indication for biopsy	Treatment prior to biopsy	Biopsy (days)	Treatment after biopsy	Follow-up (months)	Graft outcome
1	1	39	M	No	-	F 17	4	yes	no	18:25	SPKT	5		creat↑,amylase↑, proteinuria	MP	69		105.8	PDFG
2	1	42	F	No	0	F 36	0	yes	no	10:53	SPKT	4		creat↑,amylase↑	MP, ATG	41	MP	135.3	functioning
3	1	38	M	No	-	F 21	0	yes	no	15:01	SPKT	4		amylase↑, proteinuria	MP, ATG, MP	122		111.3	functioning
4	1	33	M	No	-	M 21	11	yes	no	11:35	SPKT	3		creat↑,amylase↑	MP	15		120.2	PDFG (PTLD)
5	1	30	M	No	-	F 35	0	yes	no	11:35	SPKT	5		hyperglycemia	MP, ATG	31	MP	132.9	functioning
6	1	31	F	Yes	0	M 44	56	none	no	12:41	SPKT	6	ATG	amylase↑, lipase↑		132		11.4	functioning
7	1	42	M	No	-	F 42	0	none	no	19:08	SPKT	6	ATG	peripancreatic fluid		9		18.3	functioning
8	1	32	M	Yes	-	F 16	0	none	no	14:40	PAK	3	ATG	blood counts↑, fever, edema		18	ATG x1	14.2	functioning
9	1	47	M	No	-	M 37	0	none	no	10:00	SPKT	-	Alm	peripancreatic fluid		32		3.1	functioning
10	1	32	M	No	-	M 21	25	none	no	19:30	SPKT	5	ATG	fever, edema		27		19	functioning
11	1	48	M	No	-	M 31	2	none	no	15:18	SPKT	6	ATG	amylase↑, lipase↑		915	MP	43	functioning
12	1	47	M	Yes	-	F 10	69	none	no	11:00	PAK	3	Bas	amylase↑, lipase↑		96	MP	5	functioning
13	1	37	F	Yes	0	M 16	0	none	no	26:43	PAK	5	Bas	amylase↑, lipase↑		1620	MP x2 + ATG x7	95.4	functioning
14	2	56	F	No	2	M 41	0	yes	yes	15:00	SPKT	-		protocol		319		58.8	functioning
15	2	28	F	No	1	M 31	0	none	no	13:35	SPKT	6	Bas	amylase↑, lipase↑		83	PP/IVIg x6	4.7	functioning
1	2	39	M	No	-	F 17	4	yes	no	18:25	SPKT	5		amylase↑	MP, ATG	481		105.8	PDFG
16	2	31	M	No	-	M 35	0	yes	yes	12:37	SPKT	6	OKT3	creat↑, amylase↑, hyperglycemia	MP	16	ATG	146	functioning
17	2	47	M	No	-	M 12	4	yes	yes	09:00	SPKT	3		amylase↑, HbA1c↑		169	MP, ATG, MP	75.5	functioning
18	2	46	M	No	-	M 22	5	yes	yes	12:52	SPKT	4		amylase↑	MP, ATG	222		136.6	functioning
19	3	33	M	No	-	M 49	0	yes	yes	15:40	SPKT	3		amylase↑, hyperglycemia	MP	12	ATG	57.5	PDFG (MI)
20	3	41	M	No	-	M 28	18	yes	no	11:25	SPKT	6	OKT3	creat↑,amylase↑	MP+OKT3, ATG	21		169.5	functioning
21	3	36	F	No	1	M 30	44	-	yes	05:31	SPKT	4	OKT3	amylase↑, proteinuria, edema	MP, ATG, MP	161		7.2	GL (rejection)
22	3	28	M	No	-	F 36	3	yes	no	13:30	SPKT	4		bloodglucose fluctuating	MP	29		155.7	functioning
23	3	40	M	Yes	-	M 16	25	none	no	24:52	PAK	5	ATG	amylase↑, lipase↑, hyperglycemia		174	ATG no MP, Alm	21.7	GL (rejection)
24	3	50	F	No	2	-18	65	none	no	26:30	SPKT	5	Alm	peritonitis		10		3.9	functioning
25	3	35	M	No	-	M 27	17	none	no	18:25	PTA	3	Alm	ACS		1	Pancreatectomy	0.1	GL (rejection)
26	3	36	F	No	0	M 24	4	none	no	11:00	SPKT	5	Alm	creat↑, amylase↑		35	PP/IVIg	1.9	GL (rejection)
27	3	41	F	Yes	0	M 32	64	none	no	19:41	PAK	6	ATG, Dac	leukocytosis, peripancreatic fluid		11		21.7	functioning

Table 1 | *on the previous page* | Patient and donor demographics, clinical parameters, biopsy indication and treatment in relation to pancreas allograft function.

ACS = abdominal compartment syndrome; Alm = alemtuzumab; ATG = anti-thymocyte globulin; Bas = basilixumab; biopsy (days), number of days after transplantation that biopsy was taken; CIT = cold ischemia time; creat = creatinin; Dac = daclizumab; G = group; GL = graft loss; HbA1c = hemoglobin A1c; HLA mm, human leukocyte antigen mismatches; MI =myocardial ischemia/infarction; MP = methyl prednisolone; OKT3 = muromonab-CD3; PAK = pancreas after kidney transplantation; PDFG = patient death with functioning graft; peripancreatic fluid = peripancreatic fluid collection; PP/IVIG = plasmapheresis/ intravenous immunoglobulins; PRA = panel reactive antibodies; pre-emptive = pre-emptive therapy (to be on dialysis prior to Tx); PTA = pancreas transplantation alone; PTLD = posttransplant lymphoproliferative disease; SPKT = simultaneous pancreas kidney transplantation; Tx = transplantation.

differences were found between the three groups except for a tendency to have higher PRA in group 3 and no pre-emptive therapy in group 1.

Hematoxylin and eosin staining

Fifty-six pancreas biopsies had H&E slides of which 24 of indeterminate rejection, 9 showed grade I (mild) acute cell-mediated rejection (ACR), 12 showed grade 2 (moderate) ACR and 7 showed grade 3 (severe) ACR. No chronic rejection was present in 37 pancreas biopsies, grade I chronic rejection was present in 10, grade II chronic rejection in 7, grade III chronic rejection in 1 and chronic ACR was present in 1.

Table 2 shows data on specific histomorphological lesions in 28 biopsies of patients whose DSA were determined, divided into three groups: patients with minimal or no C4d staining and no DSA were placed in group 1 (n=13), six patients (2 patients with diffuse C4d+ and absent DSA, 2 patients with focal C4d+ staining and DSA present and 2 patients with minimal or absent C4d and DSA present) were placed in group 2 and nine patients with diffuse C4d+ staining with DSA present were placed in group 3. Frequency of ACR did not differ between the groups. Between group 1 and 2, there was no significant difference in the distribution of lesions. Active septal inflammation, acinar inflammation and acinar cell injury/necrosis were present significantly more often in C4d+/DSA+ patients (group 3) than in either group 1 or 2. Group 3 differed from group 1 on additional lesions, namely ductitis (p=0.02), necrotizing arteritis (p=0.042), capillaritis (p=0.004) and edema (p=0.013). Positive C4d staining correlated significantly with the presence of ductitis, venulitis, capillaritis, acinar inflammation, acinar cell injury/necrosis and edema. In the presence of DSA, capillaritis and acinar cell injury/ necrosis were significantly found more often. Figure 2 shows the incidence of all scored histomorphological parameters.

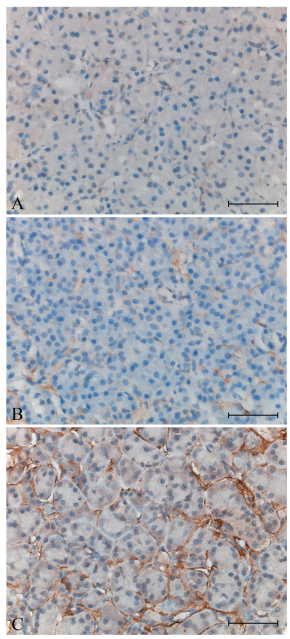


Fig 1 | C4d staining patterns. A. Negative/minimal staining; B. Focal positive staining; C. Diffuse positive staining in interacinar capillaries. Bar represents 5 µm.

C4d staining

There were 56 pancreas biopsies stained for C4d. Sixteen biopsies showed a diffuse staining pattern, 3 showed focal staining (Figure 1B) and 36 showed minimal or no staining. Of the 26 pre-transplant pancreas graft biopsies, only one showed diffuse positivity for C4d. All others showed minimal or negative C4d staining (Figure 1A).

Donor-specific antibodies

Twenty-seven patients were tested for DSA, of which 9 had anti-HLA class I, 7 had anti-HLA class II and 15 had no antibodies. Two different methods were used to assess DSA: at UMMC, Luminex was used, at the LUMC, CDC was used.

Groups and outcome

Twenty-seven patients (1 patient had 2 biopsies for a total of 28 biopsies) with C4d and DSA information were categorized into three groups (Table 2). Patients with minimal or no C4d staining and no DSA were placed in group 1 (n=13), six patients (2 patients with diffuse C4d+ and absent DSA, 2 patients with focal C4d+ staining and DSA present and 2 patients with minimal or absent C4d and DSA present) were placed in group 2 and nine patients with diffuse C4d+ staining with DSA present were placed in group 3. Figure 3A shows uncensored overall pancreas graft survival in groups 1, 2 and 3, which were 53.3%, 66.7% and 34.6%, respectively. The overall group p-value was significant for the three groups at the end of follow-up (p=0.044). Figure 3B shows censored overall pancreas graft survival in groups 1, 2 which were 100% and group 3, which was 51.9%. The overall group p-value was significant for the three groups at the end of follow-up (p=0.006). All graft failures due to rejection were found in group 3. Graft failures associated with AMR occurred during early follow-up (up to 2 years after transplantation).

DISCUSSION

In this study, we found heterogeneous C4d staining patterns in pancreas transplant biopsies taken at various time points after transplantation. In the recent Banff classification of pancreas transplant biopsies, C4d staining patterns have a role in combination with histologically proven acute rejection of the pancreas and confirmed DSA[8]. In our study, the outcomes of patients who had C4d and DSA information available were assessed. Patients with diffuse C4d positivity and concurrent DSA had poor graft survival, which was most likely related to AMR. Patients who had either DSA or C4d alone did well, similar to patients with no DSA or C4d

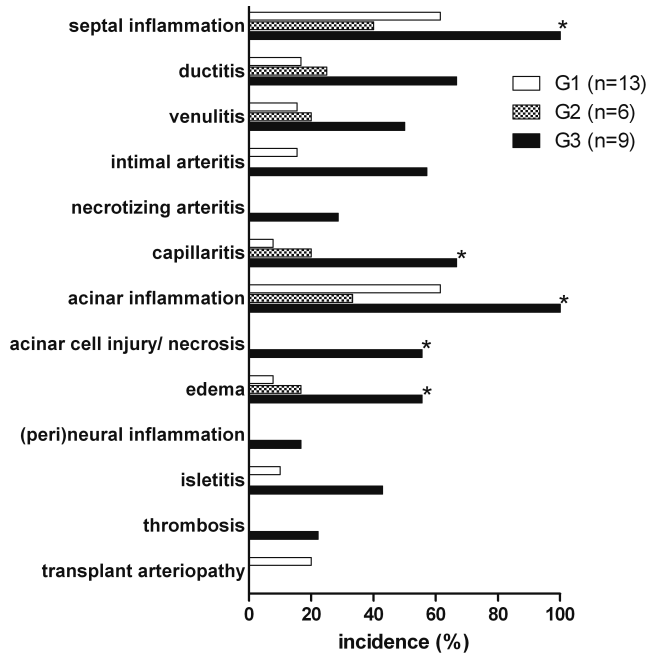


Fig 2 | Bar graph of specific histological scores as displayed in Table 2. This figure gives an indication of the abundance of various lesions, regardless of extent of the lesion. Percentages are based on adequate biopsy samples (see Table 2). The differences between groups was assessed, with * indicating a p-value of <0.05.

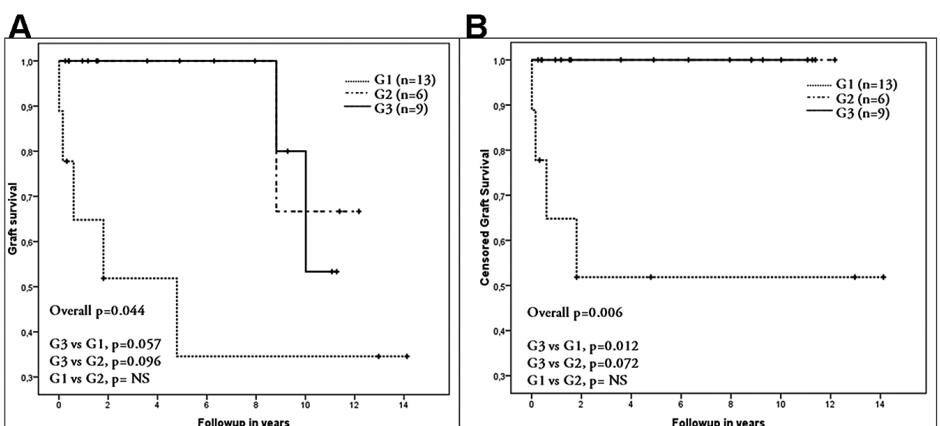
staining detected in the biopsies. Although the number of patients is relatively small, these results suggest that the occurrence of either focal C4d positivity or DSA alone are of uncertain clinical significance and may not pose additional risk of poor outcome to the pancreas graft. The possible reason for C4d positivity in the absence of HLA DSA may be due to the presence of major histocompatibility complex class I chain-related gene A (MICA) or glutathione-S-transferase T1 (GSTT1)[9], or due to the timing of DSA assessment.

C4d+ AMR following pancreatic transplantation has not been well characterized. In 2006, Melcher et al. was the first to report a patient with a SPKT who developed AMR with donor-specific HLA-DR allo-antibodies and who had positive C4d staining in the pancreas 1 month after a SPKT. The renal transplant biopsy from this patient, taken at day 10, was also positive for C4d[10]. A year later, Carbajal et al. reported a patient with pancreas after kidney transplantation (PAK) who developed AMR of the pancreas with a C4d positive biopsy. The kidney, which was from a different donor, remained unaffected, but the pancreas graft was lost[11]. In the present study, a small number of simultaneously taken kidney biopsies were available with rather heterogeneous findings in the kidney and pancreas biopsies in relation to C4d staining patterns and Banff classification. Unfortunately, DSA information was scarce in this group, and therefore, no firm conclusions could be drawn from these data. Gaber reported one case of a C4d positive surveillance biopsy

Patient No.				Active septal inflammation	Ductitis	Venulitis	Intimal arteritis	Necrotizing arteritis	Capillaritis	Acinar inflammation	Acinar cell injury/ necrosis	Edema	(Peri) Neural inflammation	Isletitis	Thrombosis	Transplant arteriopathy	Acute cell-mediated rejection	Chronic rejection
	G			0.039					0.010	0.012	0.002	0.034						
		C4d			0.004	0.036			0.001	0.005	0.002	0.004						
			DSA (HLA)					0.038		0.008								
1	1	n	n	1	-	1	0	0	0	0	0	0	-	0	0	0	Indeterminate	-
2	1	n	n	1	1	0	0	0	0	0	0	0	-	-	0	0	Indeterminate	-
3	1	n	n	1	1	0	0	0	0	1	0	0	0	-	0	3	ACR grade III (severe)	Chronic ACR
4	1	n	n	0	0	0	0	0	0	1	0	0	0	-	0	0	Normal	-
5	1	n	n	0	0	0	0	0	0	0	0	0	0	0	0	0	Normal	-
6	1	n	n	1	0	0	0	0	0	1	0	0	-	0	0	-	Normal	-
7	1	n	n	0	0	0	0	0	0	0	0	0	-	0	0	-	Normal	-
8	1	n	n	1	0	0	1	0	0	1	0	1	-	0	0	-	Normal	-
9	1	n	n	0	0	0	0	0	0	1	0	0	-	0	0	-	Normal	-
10	1	n	n	0	0	0	0	0	0	0	0	0	-	0	-	-	Indeterminate	-
11	1	n	n	1	0	0	0	0	0	1	0	0	-	0	-	-	Indeterminate	CR grade I
12	1	n	n	1	0	0	0	0	0	2	0	0	-	0	0	-	Indeterminate	-
13	1	n	n	1	0	1	1	0	1	2	0	0	-	1	0	-	ACR grade III (severe)	-
14	2	n	class I	0	0	0	-	-	0	0	0	0	-	-	0	-	Normal	-
15	2	n	class I	1	0	0	0	0	0	2	0	0	-	0	0	-	ACR grade II (moderate)	CR grade I
1	2	f	class I	0	0	0	0	0	0	0	0	0	0	-	0	0	Normal	-
16	2	f	class II	-	-	-	-	-	-	0	0	0	-	-	0	-	Normal	-
17	2	d	n	1	1	1	0	0	1	3	0	1	-	-	0	0	ACR grade II (moderate)	-
18	2	d	n	0	-	0	0	0	0	0	0	0	0	-	0	0	Inderminate	CR grade I
19	3	d	class II	1	0	1	0	0	1	3	1	1	0	0	0	0	ACR grade II (moderate)	-
20	3	d	class II	1	1	0	2	0	1	3	1	1	-	1	0	0	ACR grade I (mild)	-
21	3	d	class I	1	1	1	-	-	1	3	1	1	1	-	0	-	ACR grade I (mild)	CR grade II
22	3	d	class I	1	1	-	-	-	0	2	0	0	0	-	0	-	Normal	-
23	3	d	class I & II	1	0	0	0	0	0	2	0	0	-	0	0	0	Normal	-
24	3	d	class I & II	1	1	0	1	0	1	1	0	0	0	0	0	0	Normal	-
25	3	d	class I	1	1	1	1	1	1	3	1	1	0	1	1	0	Normal	-
26	3	d	class I & II	1	1	1	1	1	1	3	1	1	0	0	1	0	Normal	-
27	3	d	class I	1	0	0	0	0	0	1	0	0	-	1	0	0	ACR grade III (severe)	CR grade II

Table 2 | *on the previous page* | C4d, DSA, specific histological and Banff scores for pancreas graft biopsies.

Acinar cell injury/ necrosis 0 absent, 1 single cell/spotty, 2 multicellular/confluent; Acinar inflammation 0 absent, 1 focal, 2 multifocal, 3 diffuse; ACR = acute cell-mediated rejection; CR = chronic rejection; d = diffuse; DSA = donor-specific antibodies; f = focal; G = group; HLA = human leukocyte antigen; Intimal arteritis 0 absent, 1 minimal, 2 moderate to severe; n = negative; Transplant arteriopathy 0 absent, 1 present narrowing <25% lumen, 2 present narrowing 25–50% lumen, 3 present >50% lumen; – = unable to score; 0 = absent; 1 = present.



		Uncensored graft survival			Death censored graft survival		
	Total N	N of Events	N of Events		N of Events	N of Events	
			N	%		N	%
G1 (C4d and DSA negative)	13	2	11	84.6	0	13	100.0
G2 (C4d or DSA negative)	6	1	5	83.3	0	6	100.0
G3 (C4d and DSA positive)	9	5	4	44.4	4	5	55.6
all	28	8	20	71.4	4	24	85.7

Fig 3 | Uncensored (A) and censored (B) pancreas graft survival with C4d and DSA. Kaplan-meier curve and descriptive table of uncensored (A) and censored (B) pancreas graft survival in G1 patients with minimal or no C4d staining and no DSA, G2 either negative/minimal C4d or focal C4d+ staining in the presence of DSA or focal C4d+ staining in the absence of DSA, and G3 diffuse C4d+ in the presence of DSA.

taken 2 weeks after transplantation from a patient who was sensitized by two previous islet transplants and who had detectable anti-HLA antibodies[12]. Pascual et al. reported on 13 patients with an acute rejection of the pancreas, of which 2 were biopsy-proven and stained positive for C4d. In one patient, two pancreas biopsies were taken. DSA were negative at the time of the first biopsy, which was C4d positive. Three months later, a second biopsy was not only C4d positive but DSA positive as well[6]. In a recent study by Torrealba et al., the potential role of C4d in pancreas transplant biopsies in the diagnosis of AMR was described in a group of 18 patients[7]. In our study, an assessment was performed to identify specific histological lesions correlating with AMR in the pancreas, next to C4d and DSA assessment in relation to graft outcome. Although we have a relatively small number of biopsies, results from our study give an indication that septal inflammation, acinar inflammation, acinar cell injury/necrosis, capillaritis and edema are associated with AMR.

In the recent Banff classification of pancreas transplant biopsies[8], C4d staining patterns have a role in diagnosing AMR in combination with histologically proven acute rejection of the pancreas and confirmed DSA. As a clinical guideline, the results of our study emphasize the importance of taking into account a combination of C4d staining pattern, histological diagnosis and the presence of DSA before a diagnosis of AMR of the pancreas is made. The limitations of this study include the use of retrospective data and biopsies, and heterogeneity in the clinical indications for performing the transplant biopsies. The number of patients was too small to describe the clinical phenotype associated with AMR. Nevertheless, nearly all biopsies were taken within 1 year after transplantation, and the heterogeneity of the histopathological findings was helpful in establishing whether C4d could have an additional role in the work-up of pancreas transplant biopsies. The methods used to determine DSA levels in this study differed per institution, which may raise concern for the analysis and conclusions reached. Nevertheless, both methods are clinically used and accepted for diagnosis and subsequent treatment. DSA assessments were not always performed at the time of biopsy. However, AMR is a fluid state, and DSA levels are known to fluctuate over time. Therefore, it is uncertain what time point would be optimal to identify those patients developing an AMR.

In conclusion, C4d is a reliable tissue marker in combination with concurrent DSA in the evaluation of pancreas transplant biopsies. The finding that C4d positivity in pancreas transplant biopsies can occur in pancreas allografts with a good clinical outcome places doubt on the absolute value of C4d. Therefore, C4d staining patterns in pancreas transplant biopsies must be interpreted in combination with information about DSA and the histopathological lesions classified according to the Banff criteria. This clinical approach is similar to the standard procedures used to evaluate renal transplant biopsies. In this study, patients with diffuse C4d+ staining in the pancreas biopsy with concurrent DSA had poor graft survival, most likely related to AMR. The occurrence of either focally C4d+ staining or DSA poses no additional risk of a poor outcome of the pancreas graft in this study, although the sample number was relatively small. Further studies are needed to determine the value of the presence of a single marker.

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4

EARLY PANCREAS GRAFT FAILURE THROUGH ANTIBODY-MEDIATED REJECTION; A SINGLE CENTER EXPERIENCE BASED ON 256 PANCREAS TRANSPLANTATIONS

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Submitted for publication

ABSTRACT

This case-control study investigated the role of antibody-mediated rejection (AMR) in pancreas graft loss after simultaneous pancreas-kidney transplantation (SPK). Patients with pancreas graft loss in the first year post-transplantation (n=33) were compared with patients with pancreas graft function for ≥4 years (n=66). Two controls per case, one transplanted before and one after the case, were selected from all historical SPKs (n=256) performed at LUMC from 1985-2010. We investigated which pancreas grafts were lost owing to AMR, either in the absence or presence of thrombotic lesions. Early pancreas graft loss was associated with older donor age and female sex of the recipient. AMR was found in 7/33 pancreas graft losses, and characterized by the presence of *de novo* donor-specific antibodies (DSA), C4d+ staining patterns, and interacinar capillaritis as a prominent histological parameter. Eight out of 33 cases showed 2 out of 3 diagnostic components consistent with AMR. Six of these 15 cases showed pancreas graft thrombosis. DSA was tested in 27/33 cases; 8 were positive. Eight of the 33 cases showed diffuse C4d+ interacinar staining. Our findings provide evidence that AMR can cause pancreas graft loss. Early recognition of AMR might provide a means of therapeutic intervention to prevent graft loss.

INTRODUCTION

Pancreas transplantation, predominantly performed in the setting of a simultaneous pancreas-kidney transplantation (SPK), is a well-established treatment for type 1 diabetes patients with or approaching end-stage renal failure (ESRF). Patients with a SPK have a long-term survival advantage over patients with diabetes and ESRF due to diabetic nephropathy who are not transplanted and remain on dialysis[1]. Pancreas graft survival (72.6%) appears to lag behind kidney graft survival (78.5%) despite both organs being derived from the same donor[2]. Within one month post-transplantation, a marked drop in pancreas versus kidney graft survival can already be noted. After one year, the discrepancy between pancreas and kidney loss in SPK is 6.8%, and remains constant as follow-up progresses[2]. Clinically, early loss of the pancreas graft is mostly attributed to technical failure, most often implying loss through vascular thrombosis, or removal because of anastomotic leaks, bleeding, pancreatitis, or infection[3]. The first study to systematically address pancreas allograft loss described histopathological parameters, such as endothelitis and necrotizing arteritis, which seemed to point to a role for antibody-mediated rejection (AMR), although diagnostic criteria for AMR had not been defined yet at that time[4]. Currently, there is increasing interest in the role of AMR in pancreas graft loss because a consensus on diagnosis might guide therapeutic strategies[5].

The recently updated Banff schema for grading pancreas allograft rejection focuses on specific histological parameters in combination with C4d positivity of interacinar capillaries (IAC), and circulating donor-specific antibodies (DSA)[6]. In the present study, we investigated whether pancreas graft loss in SPK can be caused by AMR. Pancreas graft loss within one year of transplantation was studied in comparison with pancreas graft function in successful SPKs from all historical SPKs performed at Leiden University Medical Center (LUMC) from 1985-2010. In particular, we focused on how many pancreas grafts were lost due to AMR, either in the absence or presence of thrombotic lesions.

MATERIALS AND METHODS

Study design and data collection

This is a case-control study of pancreas allograft transplantectomies conducted at LUMC between 1985 and February 2010. A total of 256 pancreas transplantations, including SPK, pancreas after kidney (PAK), and pancreas transplant alone (PTA), were performed. Fifty-nine resulted in pancreas graft failure and transplantectomy. Forty-two patients lost their allografts <1 year. All but one specimen was retrieved from the archives of LUMC. Nine cases were excluded from the present study because the tissue only showed necrosis or extensive scarring, which was unsuitable for Banff scoring (n=4); because patients received a PAK or PTA (n=3); or because graft failure was caused by post-transplant lymphoproliferative disorder (PTLD) (n=2). This resulted in 33 SPK pancreas allograft transplantectomies <1 year after transplantation being included in this study. Two SPK recipient controls with insulin independence for ≥4 years were selected per case, one transplanted before and one

after the case. Clinical characteristics are summarized in Table 1 and shown per case in Table 2. Clinical information used for analyses was obtained from LUMC medical records after approval of the study by the Institutional Review Board.

Immunosuppressive therapy

The maintenance immunosuppressive therapy depended on the time period of transplantation: 1986-1996, prednisolone, cyclosporine A gelcaps (Sandimmune, Novartis/Basel), and azathioprine were used; 1996-2002, prednisolone, cyclosporine A microemulsion formulation (Neoral, Novartis/Basel), and mycophenolate mofetil (MMF, Roche/Basel) were used; and since 2002, prednisolone, tacrolimus, and MMF were used. In 1994, routine administration of OKT-3 was stopped after 2 cases of PTLD[7]. No induction therapy was given before 1991, or between 1994 and 1999. From 1999 onward, induction therapy consisted of either polyclonal rabbit anti-thymocyte globulin (ATG, Fresenius, Germany), an IL2 receptor blocker (daclizumab, Roche/Basel or basiliximab, Novartis/Basel), or alemtuzumab (Genzyme, Netherlands).

Acute rejection episodes (ARE) were treated according to a standard protocol consisting of methylprednisolone 1 g intravenously for 3 consecutive days for the first ARE, and a 10-day course of rabbit anti-thymocyte globulin (ATG, Merieux, initial dosage of 5 mg/kg further guided by absolute lymphocyte counts) in case of a steroid-resistant or second ARE. AMR was not recognized in any of the pancreas transplantectomies, and none of the patients received specific treatment for AMR. Matching of donor and recipient was done according to the Eurotransplant allocation rules, applying ABO blood group compatibility without prospective matching for HLA antigens. Anticoagulant therapy for all patients consisted of a prophylactic dose of heparin or, more recently, low molecular weight heparin for at least 10–14 days[8].

PRA and DSA assessment

Anti-HLA antibodies in patient sera were initially analyzed by Lambda Antigen Tray (One Lambda, USA) for ELISA HLA class I and II. The ELISA was conducted according to protocol with OD readouts at 630 nm. The sera were further tested for HLA antibody specificities by complement-dependent cytotoxicity against a panel of peripheral blood cells from 54 different donors in the absence and presence of dithiothreitol (DTT), a reducing agent that breaks down disulfide bonds in pentameric IgM but has minimal effect on IgG when used at low concentrations. The reactions were read in a semi-automatic system (Leitz, Germany) using single color readout as previously described[9]**.** Using DynaChip Antibody Analysis (Invitrogen's DynaChip® Systems, Life Technologies, UK; discontinued), the sera showing panel-reactive antibodies (PRA) >5% were analyzed for the specificity of these reactions.

(Immuno-)histochemical staining and grading

CD3, CD20, CD68 (DakoCytomation, Denmark), insulin (SantaCruz Biotechnology, Germany), and C4d (Biomedica Gruppe, Austria) immunohistochemical staining

Table 1 | Clinical characteristics of the cases and controls analyzed with binary logistic regression, both uni- and multivariate.

Clinical characteristics	Cases (n=33)	Controls (n=66)	P-value	Univariate OR	Univariate 95% CI	P-value	Multivariate OR	Multivariate 95% CI
Donor								
Age (yr; mean ± SD)	39.7 ±9.9	32 ±12.1	0.003	1.064	1.021-1.109	0.006	1.078	1.022-1.137
Gender (% male)	45.5	48.5	0.78	0.885	0.383-2.047	0.35	1.751	0.536-5.726
CIT pancreas (h; mean ± SD)	11.3 ±4.2	12 ±3.4	0.43	0.953	0.846-1.074	0.35	0.868	0.644-1.169
CIT kidney (h; mean ± SD)	12.3 ±4.2	12.9 ±3.1	0.42	0.951	0.841-1.075	0.71	1.059	0.781-1.436
Recipient								
Age (yr; mean ± SD)	41.1 ±7.8	39.3 ±7.7	0.28	1.031	0.975-1.089	0.30	1.051	0.958-1.153
Gender (% male)	48.5	65.2	0.11	0.503	0.215-1.178	0.018	0.248	0.078-0.788
Diabetes at Tx (yr; mean ± SD)	28.0 ±6.8	25.6 ±6.9	0.11	1.052	0.989-1.119	0.27	1.054	0.960-1.157
HLA-class 1 MM (mean ± SD)	4 ±2.7	4 ±2.9	0.32	0.793	0.504-1.250	0.45	0.798	0.433-1.437
HLA-class 2 MM (mean ± SD)	2 ±1.3	2 ±1.3	0.54	0.800	0.391-1.640	0.60	1.278	0.508-3.216
Peak PRA (%; median [IQR])	12 [2-8.5]	9 [2-8]	0.41	1.010	0.987-1.033	0.24	1.019	0.988-1.051
Transplantation								
Pre-emptive (% yes)	33.3	42.4	0.38	1.474	0.616-3.528			
Drainage (% bladder)	84.4	92.4	0.23	0.259	0.604-8.456			
Induction therapy (% yes)	48	62	0.20	0.574	0.247-1.336			
Maintenance AZA vs MMF	17/16	33/33	0.89	1.062	0.461-2.451			
Maintenance Tac vs CsA	11/22	22/44	1.00	1.000	0.412-2.426			
Nr of rejections (mean ± SD)	1.6 ±1.4	1.6 ±1.4	0.96	1.008	0.739-1.376			

For normally-distributed data, the mean with standard deviation (SD) is expressed; for data not normally distributed, the median and interquartile range (IQR) is expressed. Abbreviations: AZA = azathioprine; CI = confidence interval; CIT = cold ischemic time; CsA = cyclosporine; drainage = exocrine fluid drainage, either bladder or enteric; HLA = human leukocyte antigen; maintenance = maintenance immunosuppressive therapy; MM = mismatch; MMF = mycophenolate mofetil; nr = number; PRA = panel reactive antibodies; OR = odds ratio; Tx = transplantation; Tac = tacrolimus.

#		Age at Tx	Sex	DM at Tx	Pregnancy	Donor sex, age	HLA Ab	Preemptive	CIT (hr)	Drainage type	HLA mm	Ind. therapy	Maintenance therapy	Rej. times	Indication for pancreas transplantectomy	FU pancreas (months)	FU kidney (months)	FU patient (years)
1	1	34.1	M	17	N/A	F 46	-	Y	5.0	bladder	3	-	CI+AZA+P	2	thrombosis/infarction	0.1	159.6	23.3
2	1	35.8	M	22	N/A	F 45	-	Y	14.2	bladder	5	-	CI+AZA+P	2	thrombosis/infarction	0.0	229.4	19.1
3	1	34.5	F	31	?	M 44	-	N	17.0	bladder	4	OKT3	CI+AZA+P	2	thrombosis/infarction	0.1	174.8	16.7
4	1	57.7	M	26	N/A	M 44	-	Y	14.1	bladder	4	-	CI+AZA+P	2	infection	1.4	1.4	1.4
5	1	50.3	M	40	N/A	M 32	-	Y	7.2	bladder	3	-	CI+AZA+P	2	rejection (acute/chronic)	0.2	70.0	10.7
6	1	35.0	F	25	no	M 33	-	N	11.9	bladder	6	-	CI+MMF+P	3	thrombosis/infarction	8.4	0.4	1.3
7	1	46.2	M	29	N/A	F 43	-	Y	7.3	bladder	6	ATG	CI+MMF+P	2	thrombosis/infarction	0.2	66.0	5.5
8	1	48.5	M	28	N/A	M 41	-	N	16.8	bladder	5	ATG	CI+MMF+P	0	thrombosis/infarction	0.1	85.2	7.1
9	1	40.2	F	24	no	M 18	-	N	6.0	bowel	5	ATG	CI+MMF+P	0	thrombosis/infarction	6.2	103.7	8.6
10	1	51.0	M	34	N/A	F 43	-	Y	9.1	bladder	5	ATG	T+MMF+P	0	thrombosis/infarction	0.1	89.0	7.4
11	1	43.2	F	39	?	F 37	-	N	14.6	bladder	3	ATG	T+MMF+P	0	thrombosis/infarction	0.5	64.5	5.4
12	1	37.2	F	24	?	F 50	-	N	9.0	bladder	4	ATG	T+MMF+P	1	thrombosis/infarction	0.2	70.8	5.9
13	1	41.7	M	28	N/A	F 21	-	Y	13.8	bladder	3	ATG	T+MMF+P	2	thrombosis/infarction	0.1	46.2	3.9
14	1	43.7	F	36	?	M 20	-	N	15.2	bowel	6	ATG	T+MMF+P	0	thrombosis/infarction	0.1	45.2	3.8
15	1	28.4	M	23	N/A	F 52	-	N	-	-	4	-	CI+AZA+P	1	thrombosis/infarction	1.1	0.7	0.2
16	1	43.3	M	27	N/A	M 20	?	Y	4.9	bladder	3	-	CI+AZA+P	3	thrombosis/infarction	0.0	7.6	4.4
17	1	48.6	M	27	N/A	M 53	-	N	-	bladder	3	-	CI+AZA+P	0	rejection (acute/chronic)	0.0	0.0	2.1
18	1	42.4	F	34	yes	M 45	+	N	14.3	bladder	3	-	CI+AZA+P	0	technical problems	1.1	1.1	0.1
19	2	42.1	M	29	N/A	F 51	-	N	5.3	bladder	3	-	CI+AZA+P	4	rejection (acute/chronic)	5.2	8.2	2.4
20	2	52.7	F	21	no	F 46	-	Y	15.8	bladder	2	-	CI+AZA+P	2	thrombosis/infarction	0.0	96.5	8.0
21	2	36.3	M	24	N/A	M 46	-	N	9.6	bladder	4	ATG	CI+MMF+P	1	thrombosis/infarction	0.1	97.0	9.1
22	2	42.1	F	37	yes	F 49	-	N	12.4	bladder	4	ATG	T+MMF+P	1	thrombosis/infarction	0.1	78.9	6.6
23	2	46.5	F	38	yes	F 43	-	N	15.3	bladder	1	ATG	T+MMF+P	2	thrombosis/infarction	0.1	0.4	6.1
24	2	44.4	F	37	?	M 48	-	N	12.4	bladder	4	ATG	T+MMF+P	3	thrombosis/infarction	0.3	48.6	4.1
25	2	41.0	F	29	yes	F 43	-	N	10.8	bowel	5	ATG	T+MMF+P	0	technical problems	0.5	1.2	0.1
26	2	49.4	M	37	N/A	F 35	-	N	14.0	bowel	6	ATG	T+MMF+P	0	thrombosis/infarction	0.1	43.3	3.6
27	3	33.1	F	25	?	F 50	-	N	16.1	bowel	5	ATG	T+MMF+P	0	thrombosis/infarction	0.1	99.6	8.3
28	3	30.4	M	26	N/A	F 35	-	N	5.3	bladder	3	-	CI+AZA+P	3	rejection (acute/chronic)	2.4	3.5	8.6
29	3	31.4	F	17	?	M 24	-	Y	4.5	bladder	4	-	CI+AZA+P	1	rejection (acute/chronic)	1.2	0.2	4.8
30	3	35.9	F	18	?	M 35	-	N	15.0	bladder	4	-	CI+AZA+P	4	rejection (acute/chronic)	4.9	3.8	4.2
31	3	36.4	M	28	N/A	F 41	-	N	12.1	bladder	4	-	CI+AZA+P	4	rejection (acute/chronic)	3.5	2.2	4.2
32	3	23.2	F	14	yes	F 41	+	N	6.6	bladder	3	-	CI+AZA+P	4	rejection (acute/chronic)	2.5	2.5	1.4
33	3	49.2	F	29	yes	M 40	-	Y	16.2	bladder	4	OKT3	CI+AZA+P	3	rejection (acute/chronic)	6.2	10.4	16.1

Table 2 | *on the previous page* | Patient and donor demographics, clinical parameters, transplantectomy indication, and treatment split into 3 groups based on C4d, DSA and histology assessments.

Abbreviations: ATG = anti-thymocyte globulin; biopsy (days) = number of days after transplantation that biopsy was taken; CI = cyclosporine ; CIT = cold ischemia time; F = female; G = group; HLA Ab = pre-transplantation non-donor-specific human leukocyte antigen antibodies; HLA mm = human leukocyte antigen mismatches; M = male; MMF = mycophenolate mofetil; .N/A = not applicable; NC = no category; OKT3 = muromonab-CD3; pre-emptive = pre-emptive therapy (to be on dialysis prior to Tx); rej. Times = number of rejections; T = tacrolimus; Tx = transplantation
G1 = AMR negative/requires exclusion; G2 =consistent with AMR; G3 = acute AMR

was performed on formalin-fixed, paraffin sections of pancreas transplantectomies. Additionally, C4d staining was performed on a select number of renal and duodenal tissue slides from specimens obtained at the time of pancreas transplantectomy (detailed staining protocols in supplemental data, Figure S1).

Hematoxylin and eosin (H&E), Congo red, and C4d stained pancreas transplantectomies were scored according to the Banff 2011 grading scheme[6] (Table 3). CD3, CD20, and CD68 staining patterns were graded by I.B. and H.d.K together on a 0 to 3+ scale.

Statistical analyses

Raw data were processed using descriptive statistics and graphical representations. All clinical characteristics comparing cases and controls were analyzed with binary logistic regression, either univariate or forced-entry multivariate. For normally-distributed data, the mean with standard deviation (SD) is expressed; for data not normally distributed, the median and interquartile range (IQR) is expressed. Histological parameters within the cases were analyzed with two-tailed Fisher exact test if binary, or Kendall's Tau-b statistic using exact significance testing if ordinal. The Kaplan-Meier product limit method was used to estimate the pancreas allograft survival times for SPK patients who lost their pancreas graft within 1 year after transplantation, and a one-minus survival curve was plotted. Significance was set at $p \leq 0.05$ and calculated with SPSS 16.0 (SPSS, USA).

RESULTS

Clinical characteristics

The mean follow-up time after SPK of the 99 recipients was 9.2 (SD ±5.8) years. At the time of transplantation, recipients were 39.9 (SD ±7.7) years old, with a documented

history of type 1 diabetes for 26.4 (SD ±7.0) years (Table 1). In multivariable analysis, donor age and female gender were significantly associated with early pancreas graft loss. The cumulative incidence of pancreas graft loss <1 year post-transplantation is plotted in Figure 1. Early pancreas graft loss occurred in 21 (64%) patients within a month after transplantation; 18 (86%) of those lost their pancreas grafts within the first week.

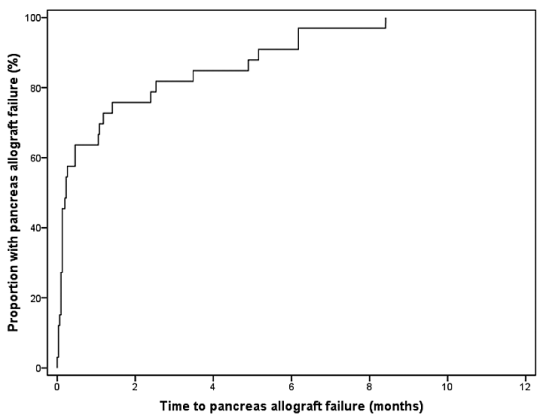

Fig 1 | Time to pancreas allograft failure in SPK patients who lost their pancreas within one-year after transplantation. Kaplan–Meier one minus survival plot for time (months) to pancreas allograft failure in the 33 SPK patients which lost their pancreas allograft within 1 year after transplantation in the period 1985 to February 2010.

DSA, C4d, and AMR

Before transplantation, none of the 99 recipients had DSA. After pancreas transplantectomy, DSA were found in 8/27 (30%) tested cases, of which 5 were female recipients. Of the DSA-positive cases, 5 showed only anti-HLA class II DSA and 3 showed both anti-HLA class I and class II DSA positivity (Table 3).

Before transplantation, non-donor-specific anti-HLA class I antibodies were found in the case group in 3/32 (9%) recipients, all of whom were females. After transplantation, non-donor-specific anti-HLA class I antibodies were found in 2/27 (7%) subjects, both female recipients. One of the two had the same non-donor-specific anti-HLA antibodies before and after transplantation, and in the other only post-transplantation non-donor-specific anti-HLA class I antibodies were identified.

C4d staining of the IAC in the resected pancreas allograft was negative in 19/33 (58%), focally positive in 6/33 (18%), and diffusely positive in 8/33 (24%) cases. To investigate C4d staining patterns of the duodenum 23 specimens from 33 rejected grafts were retrieved. C4d staining of capillaries and small vessels, both in the mucosa, submucosa and muscle of the resected duodenum was negative in 16/23 (69%), focally positive in 5/23 (22%), and diffusely positive in 2/23 (9%) duodenums of pancreas transplantectomy cases. These cases seemed to show a positive staining pattern in clusters of capillaries, but the percentage of positive vessels never exceeded 10% of all capillaries. In 16/23 (69%) cases C4d staining patterns in the duodenum were similar to those in the pancreas (Table 3).

Following the Banff 2011 grading schema[6], cases were classified into 3 groups: 18/33 (55%) cases had either 0 or 1 out of the 3 diagnostic components of acute AMR (circulating DSA, ≥5% of IAC C4d+, and morphological evidence of tissue injury), requiring exclusion of AMR (Group 1). In 8/33 (24%) cases, 2 out of the 3 diagnostic components were present, classifying the cases as consistent with acute AMR (Group 2). In the remaining 7/33 (21%) cases, acute AMR of the pancreas was present at the time of transplantectomy showing 3 out of 3 diagnostic components (Group 3).

In the control group, 8 patients lost their graft 8.7 [IQR 4.4-10.5] years after transplantation. Three pancreas transplantectomy specimens could be retrieved from our archives showing chronic AMR (n=1), TCMR grade 2 (n=1), and chronic allograft rejection/graft fibrosis stage 3 (n=1). Duodenal C4d staining patterns in these control cases were inconsistent with pancreas C4d staining patterns.

Histology and pancreas allograft failure

Tissue slide examinations according to the Banff 2011 grading schema[6] of the lost pancreas grafts in the 33 cases showed the following: in group 1, 8/18 (44%) patients had pure TCMR and 10/18 (56%) had no rejection. In group 2, 3/8 (37%) patients had only hallmarks consistent with AMR, 5/8 (63%) showed mixed TCMR and features consistent with AMR. In 2 of the latter group chronic changes were found. In group 3, 3/7(43%) showed pure acute AMR, in 1 case chronic active AMR was also found. Mixed rejection was found in 4/7 (57%), showing both acute AMR and TCMR hallmarks, in 1 case together with chronic stages of mild graft fibrosis (Table 3).

Histologically proven thrombotic lesions were present in 28/33 (85%) pancreas grafts. Loss of these grafts was clinically attributed to thrombosis in 21/33 (64%). Of AMR group 3 (Table 2&3), 6 had been clinically diagnosed as loss due to rejection and 1 loss was clinically attributed to thrombosis. Histological analyses identified signs of thrombosis in 5/7 (71%).

Specific immunohistochemical markers were compared to AMR, C4d, DSA and increasing stages of TCMR, to investigate whether these could add to the diagnostic and pathophysiologic data (not shown). The presence of DSA was associated with increased CD3 staining (p=0.005). All three markers were found to be significantly increased with increased TCMR grading (CD3 p=0.005, CD20 p=0.003, and CD68 p=0.018).

Of the clinical characteristics within the case group, only PRA >5% pre-transplantation was associated with C4d staining (p=0.001) and DSA (p=0.027). Patients with increasing stages of AMR or with *de novo* DSA detected after transplantectomy had experienced more rejection episodes (p=0.036; p=0.001 respectively).

Islet pathology[6] was analyzed in this cohort, albeit correlation with recurrence of autoimmune diabetes or loss of glycemic control could not be assessed. Amylin deposition in islets, thought to be associated with loss of glycemic control, was never found. ß-cell loss assessed by insulin staining was observed, but in no apparent correlation with other markers (Table 3). Specific C4d staining within the microvasculature of the islet was observed in 10 cases, in which 5/7 (71%) were found in group 3, acute AMR.

Ptn #	G	C4d	DSA (HLA)	Active septal inflammation	Ductitis	Venulitis	Intimal arteritis	Necrotizing arteritis	Capillaritis	Acinar inflammation	Acinar cell injury/ necrosis	(Peri-) neural inflammation	Transplant arteriopathy	CD3	CD20	CD68	β-cell loss	Islet C4d	Duodenum C4d	Acute cell-mediated rejection	Chronic rejection
1	1	n	n	0	0	0	0	0	0	1	2	-	0	1	0	2	-	-	n/a	Normal	not present
2	1	n	n	0	1	0	0	0	0	0	2	0	3	1	3	1	++	-	n	TCMR grade 1 (mild)	-
3	1	n	n	0	0	0	0	0	0	0	0	0	0	0	0	2	-	-	n	Normal	not present
4	1	n	n	0	0	0	0	0	0	0	0	0	0	1	1	1	+	-	f	Normal	not present
5	1	n	n	1	1	1	0	0	0	2	2	0	0	2	1	3	-	-	n	TCMR grade 1 (mild)	not present
6	1	n	n	1	1	0	1	0	0	3	1	1	0	3	3	3	-	-	n	TCMR grade 2 (moderate)	not present
7	1	n	n	1	-	1	1	0	0	2	0	1	0	2	0	2	-	-	n	TCMR grade 2 (moderate)	not present
8	1	n	n	0	0	0	0	0	0	0	0	0	0	1	0	2	-	-	n/a	Normal	not present
9	1	n	n	1	-	1	0	0	0	3	2	1	0	1	0	3	n/a	-	n	Normal	not present
10	1	n	n	0	0	0	1	0	0	0	2	0	0	1	0	1	++	-	f	Normal	not present
11	1	n	n	1	1	1	2	0	1	3	2	0	0	1	2	2	-	+	n	TCMR grade 3 (severe)	not present
12	1	n	n	1	-	1	0	0	0	1	0	0	0	3	3	3	+	-	n/a	TCMR grade 1 (mild)	not present
13	1	n	n	0	0	1	0	0	0	0	0	0	0	1	0	1	+	-	n	Normal	not present
14	1	n	n	1	1	1	0	0	0	1	0	0	0	0	0	2	-	-	n/a	TCMR grade 1 (mild)	not present
15	1	n	not tested	1	1	0	0	0	0	2	0	0	0	1	2	2	++	-	n/a	TCMR grade 1 (mild)	not present
16	1	n	not tested	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-	n	Normal	not present
17	1	n	not tested	0	0	1	0	0	0	0	0	0	0	0	0	0	-	+	f	Normal	not present
18	1	n	not tested	0	0	0	0	0	0	0	0	0	0	0	0	1	-	+	n	Normal	not present
19	2	n	class II	1	1	0	0	0	1	3	1	1	0	2	3	2	-	-	n/a	TCMR grade 1 (mild)	grade I
20	2	f	n	0	0	0	0	0	0	0	2	0	0	0	0	2	-	-	n/a	Normal	not present
21	2	f	n	0	0	0	1	0	1	0	2	0	1	0	0	2	+	-	n	TCMR grade 2 (moderate)	chronic TCMR
22	2	d	n	1	1	1	1	1	0	2	2	0	0	0	1	1	-	+	n	Normal	not present
23	2	d	n	0	0	1	0	0	0	0	2	0	0	0	0	2	-	-	n	Normal	not present
24	2	d	n	1	0	1	0	0	1	3	0	0	0	1	1	2	-	-	n/a	TCMR grade 1 (mild)	not present
25	2	f	not tested	1	1	0	0	0	0	1	0	0	1	1	1	2	-	+	f	TCMR grade 1 (mild)	not present
26	2	d	not tested	1	0	0	1	0	0	0	0	0	0	2	0	3	-	-	n	TCMR grade 2 (moderate)	not present
27	3	f	class II	1	0	0	1	0	0	0	0	0	0	1	0	1	-	+	n	TCMR grade 2 (moderate)	not present
28	3	f	class II	1	1	1	1	0	1	3	1	1	0	2	3	2	-	+	d	TCMR grade 2 (moderate)	grade I
29	3	f	class I&II	1	-	0	2	1	1	3	2	1	0	3	3	3	-	+	f	TCMR grade 2 (moderate)	not present
30	3	d	class II	1	1	0	2	1	1	3	2	1	0	3	2	3	++	+	n/a	TCMR grade 3 (severe)	not present
31	3	d	class I&II	1	-	0	0	0	1	3	2	0	0	2	0	-	+	-	n/a	Normal	not present
32	3	d	class II	1	0	1	1	0	1	0	0	1	0	2	1	2	-	+	d	Normal	not present
33	3	d	class II	0	-	0	0	0	1	3	2	1	0	2	0	3	++	-	n	Normal	chronic active AMR

Table 3 | *on the previous page* | C4d, DSA, and specific histological and Banff scores for pancreas grafts transplantectomies.

Acinar cell injury/necrosis; 0 = absent, 1 = single cell/spotty, 2 = multicellular/confluent. Acinar inflammation; 0 =absent, 1 = focal, 2 = multifocal, 3 = diffuse. TCMR = T-cell mediated rejection; AMR = antibody-mediated rejection; d = diffuse; DSA = donor-specific antibodies; f = focal; G = group; HLA = human leukocyte antigen. Intimal arteritis; 0 = absent, 1 = minimal, 2 = moderate to severe. n = negative; NC = no category. Transplant arteriopathy; 0 = absent, 1 = present narrowing <25% lumen, 2 = present narrowing 25–50% lumen, 3 = present >50% lumen; – = unable to score; 0 = absent; 1 = present.
G1 = AMR negative/requires exclusion; G2 =consistent with AMR; G3 = acute AMR.

We also analyzed concurrent renal transplant pathology in our cases where available. In 13/33 pancreas transplantectomy cases, a renal transplantectomy was retrieved from our archives. Seven had histomorphological lesions consistent with (suspected) AMR and were found in all 3 pancreas AMR categories, but predominantly (4 cases) in group 3 (acute AMR of the pancreas). From 6 patients in pancreas AMR group 3 (Table 2&3), concurrent renal transplant biopsies were taken at the time of pancreas transplantectomy showing a concurrent diagnosis of AMR (n=5), a concurrent diagnosis of TCMR (n=2). In 3/6 renal biopsies, thrombotic lesions were found. These 6 kidney allografts were lost <1 year post-transplantation. The anti-HLA class II DSA 7th pancreas AMR loss had no concurrent renal tissue specimen and this graft functioned for over 8 years.

DISCUSSION

This study is the first to show that early loss of pancreas grafts can be caused by AMR, defined as diffuse C4d-positive staining in the presence of DSA, and specific histological parameters. In this study, 7 cases had pancreas graft loss due to AMR of which 5 showed thrombosis of the graft and of which 4 had previously been diagnosed with acute cellular rejection. The importance of identifying pancreas graft loss caused by AMR is illustrated by the 7 patients with AMR in their pancreas grafts of whom 6 also lost their kidney grafts <1 year after transplantation.

In this study, we used pancreas graft loss within 1 year as a starting point to search for AMR as a possible cause for early graft loss. All but one of the actual AMR cases, defined according to Banff criteria, were transplanted between 1988 and 1992. During this period, induction therapy was not a standard part of the initial immunosuppression at our center. Additionally, over time maintenance immunosuppression evolved from steroids with cyclosporine gelcaps and azathioprine to the more potent combination of tacrolimus and MMF. One case was

transplanted in 2002 receiving ATG induction therapy and maintenance immunosuppresion of prednisolone, tacrolimus, and MMF. It is possible that the change in therapy regimen reduced pancreas graft loss due to AMR. In a recent study, we showed that AMR in the pancreas may occur, but need not lead to immediate graft loss[10].

This study also underlines differences in pancreas and kidney allograft behavior from the same donor in SPK recipients with respect to both the occurrence of AMR and graft survival. Pancreas loss in the majority of patients was due to thrombosis within the first week after transplantation in the absence of AMR, with subsequent good survival of the kidney allograft. The cause of thrombosis in these cases was most likely non-immunological, and correctly designated as 'lost due to technical failure' on clinical grounds. It is relevant to note that 6/7 acute AMR cases identified retrospectively in this study, lost their pancreas beyond the first month after transplantation. Although thrombosis was present in most of these grafts, it was not as extensive as in grafts lost shortly after transplantation, pointing towards another pathophysiological mechanism. In the context of inflammation and stress, the upregulation of HLA class II antigens on endothelial cells[11] and the relative low vascular flow state may make a pancreatic graft more prone to thrombosis. Notably, the DSA-positive cases in our study all had antibodies against HLA class II, either with or without antibodies against HLA class I.

DSA were associated with an increased number of CD3 positive-stained cells. In the current study, CD3, CD20, and CD68 positively-stained cells were significantly more abundant in increasing stages of TCMR. Although worth mentioning, this most likely does not reflect a useful additional marker for diagnosing TCMR in pancreas allografts as this can easily be distinguished by morphological features. Furthermore, DSA and C4d were associated with a PRA of >5%. AMR and DSA were associated with an increased number of rejection episodes as well, most likely reflecting the time it takes after transplantation for *de novo* antibodies to form. This time-span allows for the occurrence of more rejection episodes prior to graft failure and transplantectomy.

Duodenal patch biopsies have been proposed for diagnosing rejection of the pancreas allograft[12]. In only 69% of cases did C4d staining patterns of duodenum and pancreas from the same donor correspond. In descriptions of renal and pancreas C4d capillary staining in SPK, this discordance is well-established[10;13;14]. Thus, in this study on pancreas transplantectomies we did not find grounds to use duodenal allograft nor kidney allograft tissue as a surrogate for pancreas allograft tissue.

This study shows that AMR can cause early pancreas graft loss, and grafts lost through AMR are likely to show thrombotic lesions. In this cohort of SPK recipients, AMR of the pancreas allograft most often occurred more than 1 month post-transplantation. In 3 cases the simultaneously transplanted kidney graft was lost before the pancreas, 1 kidney was lost at the same time, and in 3 cases the kidney was lost after the pancreas graft. DSA development appears the most characteristic feature of AMR diagnosis and is associated with rejection episodes. Therefore, DSA monitoring after transplantation in combination with histological assessment of the pancreas allograft may help to further improve outcomes after SPK transplantation.

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SUPPLEMENTAL DATA

Staining protocol

Sections were deparaffinized; antigen retrieved with TRIS/EDTA, pH 9.0 (except insulin); and sections blocked for endogenous peroxidase. Primary antibodies were applied for 1 hour at room temperature with a 1:300 dilution of monoclonal mouse anti-human CD3 antibody clone F7.2.38, a 1:800 dilution of monoclonal mouse anti-human CD20cy antibody clone L26, a 1:1000 monoclonal mouse anti-human CD68 antibody clone KP1, 1:100 dilution of rabbit anti-insulin polyclonal antibody (H-86) overnight, and a 1:25 dilution of rabbit anti-C4d polyclonal antibody[10], respectively. Primary antibody binding was visualized with the REAL™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (DakoCytomation, Denmark) according to the protocol. All stained sections were counterstained with hematoxylin.

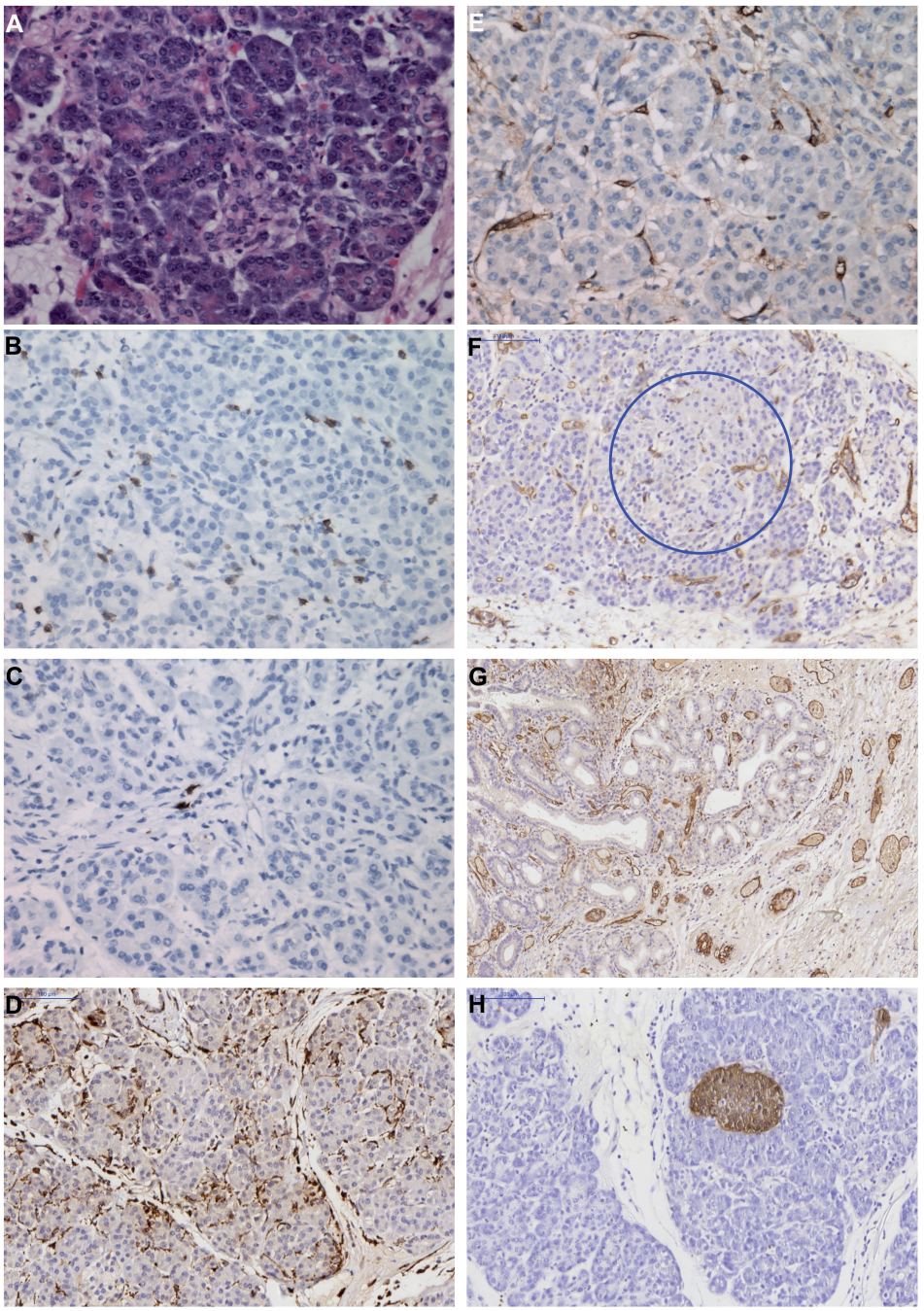

Fig S1 | (Immuno-) staining of patient 32 (Table 2&3). (A) H&E staining, (B) T-cell (CD3) staining, (C) B-cell (CD20) staining, (D) monocyte and macrophage (CD68) staining, (E) diffuse interacinar capillary C4d staining pattern on pancreas, (F) C4d staining in inter-islet capillaries (blue circle around islet of Langerhans), (G) diffuse C4d staining of capillaries and small vessels, both in the mucosa, submucosa and muscle of the resected duodenal patch, (H) insulin staining of ß-cells in islet of Langerhans.

5

ISLET TRANSPLANTATION IN TYPE 1 DIABETES

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British Medical Journal 2011;342:d217

SUMMARY POINTS

- Islet of Langerhans transplantation is used in a select group of patients with type 1 diabetes with severe glycaemic lability, recurrent hypoglycaemia, and hypoglycaemia unawareness
- The procedure is minimally invasive, with few procedure related complications
- Two to three islet infusions are usually needed to achieve insulin independence
- Most patients need insulin by five years post-transplantation owing to declining graft function; beneficial effects on the frequency of hypoglycaemic episodes and hypoglycaemia awareness remain
- Most long term complications are related to systemic immunosuppression
- The risk-benefit ratio of islet transplantation should be carefully weighed by the treating physician and the potential recipient, who should be given adequate information

SOURCES AND SELECTION CRITERIA

We searched PubMed, Embase, Web of Science, Cochrane, CINAHL, Academic Search Premier, and ScienceDirect using the keyword "islet transplantation". We limited our search to the English language and to human studies. We found no randomised controlled trials, and most publications lacked an appropriate control group that was intensively managed by insulin using modern treatment regimens. Data were mainly derived from case series, follow-up studies, crossover studies, and small trials. We also consulted published reviews and expert knowledge if considered necessary.

A clinical review in the *BMJ* in 2001 anticipated that by 2010 transplantation of islets of Langerhans would be the treatment of choice for most patients with type 1 diabetes[1]. Currently, islet transplantation is an option for a specific group of patients with type 1 diabetes only—those with severe glycaemic lability, recurrent hypoglycaemia, and hypoglycaemia unawareness. Patients with type 1 diabetes—who must deal with daily subcutaneous insulin injections, regular finger pricks for glucose measurements, and worries about hypoglycaemic episodes and long term complications of diabetes, hope for a cure for their disease and may ask their doctors about islet transplantation. Therefore, doctors who treat such patients should understand the potential benefits of islet transplantation as well as the hurdles that need to be overcome before it is widely used (box 1).

Box 1 | What general practitioners need to know.

- Most patients with type 1 diabetes do not fit the criteria for islet transplantation
- It is not a treatment option for patients with type 2 diabetes, who usually have insulin resistance and considerable remaining islet function
- Patients who have undergone successful islet transplantation usually have greatly improved hypoglycaemia awareness and experience fewer hypoglycaemic episodes
- Although insulin independence can be achieved, most patients will ultimately have to resume insulin treatment, but the frequency of hypoglycaemic episodes remains reduced
- Islet transplantation can improve glycaemic control and reduce risk of progression of vascular complications
- The clinical problems related to long term use of immunosuppressive agents include drug interactions, infections, and an increased risk of certain cancers

WHY ISLET TRANSPLANTATION?

Type 1 diabetes is caused by the autoimmune destruction of insulin producing ß cells in the pancreatic islets of Langerhans. A well defined worldwide population based survey showed that the incidence of childhood onset type 1 diabetes is rising rapidly, with an overall annual increase of 3.4% between 1995 and 1999[2]. A multicentre prospective registration study from Europe predicted that the number of prevalent cases of type 1 diabetes in children below the age of 15 will increase by 81% from 18 500 in 2005 to 33 500 in 2020 in the United Kingdom[3]. For patients with type 1 diabetes, exogenous insulin administration to control blood glucose is a lifesaving treatment, but it also has a negative impact on personal and social functioning, not least because of the daily risk of hypoglycaemic episodes. In addition, normoglycaemia cannot be achieved by exogenous insulin and secondary complications such as retinopathy, neuropathy, nephropathy, and

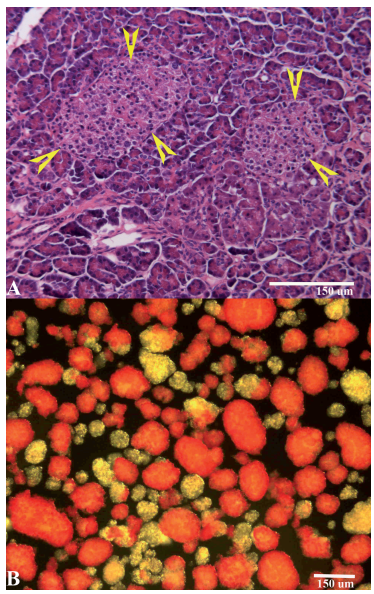

Fig 1 | (A) Histological section showing two islets (yellow arrows) in the pancreas. (B) Isolated islets stain red with dithizone; non-islet (exocrine) tissue is yellow. Image B courtesy of Marten Engelse, Human Islet Isolation Facility, Leiden University Medical Centre, Netherlands

cardiovascular disease occur despite good glycaemic control[4;5]. Consequently patients with type 1 diabetes face living with the long term debilitating consequences of their disease.

Pancreatic islets constitute only 1-2% of the pancreas. They consist of clusters of mainly hormone producing cells (fig 1), with insulin producing ß cells being the most abundant cell type[6]. Replacement of ß cells is the only treatment capable of normalising glycaemia without the risk of hypoglycaemia because ß cells respond to changes in glucose concentrations by subtly adjusting insulin secretion to maintain glucose homoeostasis.

Whole pancreas transplantation, a form of ß cell replacement that has been performed since 1966, is a major surgical procedure with considerable peri-transplant complications and post-transplant morbidity related to the transplantation of superfluous exocrine pancreatic tissue. Islet transplantation, however, is minimally invasive and has low morbidity because the islets are infused percutaneously via a catheter into the hepatic portal vein. Figures 2 and 3 illustrate the complex processes of islet isolation and transplantation.

WHO IS ELIGIBLE?

Islet transplantation has not become a mainstream treatment for type 1 diabetes largely because of a shortage of (high quality) donor organs for islet isolation, the high costs of isolation procedures and maintenance of a specialised human islet isolation laboratory, and the need for lifelong use of immunosuppressive agents. Islet transplantation is therefore usually reserved for a highly selected group of patients with severe glycaemic lability, recurrent hypoglycaemia, and a reduced ability to sense symptoms of hypoglycaemia (reduced hypoglycaemia awareness). A cross sectional Danish-British multicentre survey found that patients with type 1 diabetes have an average of 1.3 severe hypoglycaemic episodes per patient year[7]. However, the distribution was highly distorted, with about 5% of patients accounting for 54% of all reported episodes. Because islet transplantation improves recipients' hypoglycaemia awareness and reduces the frequency of hypoglycaemic episodes in the long term, this subgroup of patients would probably benefit most from the procedure. Islet transplantation is not a treatment option for type 2 diabetes, which is caused mainly by insulin resistance, with patients usually having considerable remaining islet function.

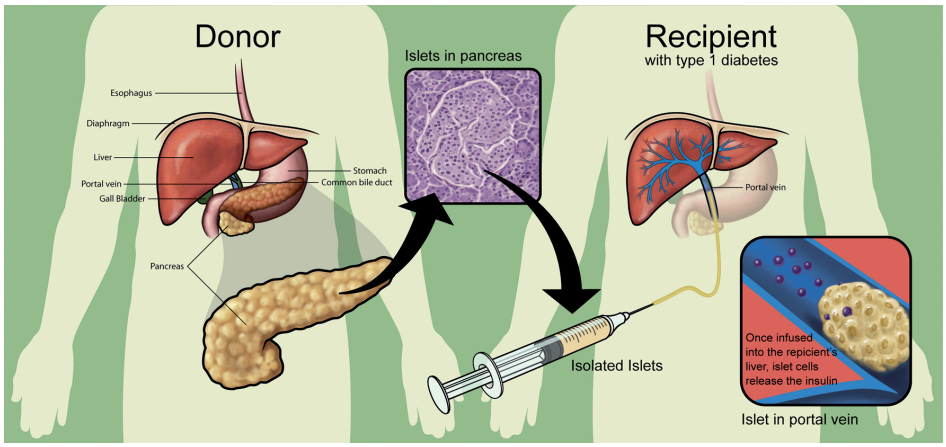

Fig 2 | Process of clinical islet transplantation for the treatment of type 1 diabetes (adapted from Naftanel and Harlan[38])

Most patients who undergo islet transplantation participate in clinical research studies with varying inclusion criteria. Inadequate glycaemic control with recurrent hypoglycaemia is the entry criterion most often used. However, because microvascular and perhaps macrovascular complications have stabilised in some recipients of islet transplantation, studies that focus on microvascular complications and inadequate glycaemic control rather than hypoglycaemia related problems have begun. A retrospective cohort study found that islet transplantation may also prolong the survival of a previous kidney graft[8]. For these patients, who already receive immunosuppressive agents, the clinical decision to perform islet transplantation is influenced by a different risk-benefit ratio. In the UK, islet transplantation is now funded by the NHS and is particularly indicated for patients with reduced hypoglycaemia awareness or those taking immunosuppressive drugs because of a previous kidney transplant.

HOW DO WE DEFINE SUCCESS OF ISLET TRANSPLANTATION?

Observations from long term studies triggered a debate about how to define the "success" of islet transplantation. Historically, the primary goal of islet transplantation has been the ability of donor islets to maintain normal glucose control and removal of the need for exogenous insulin. "Insulin independence" is a comprehensible clinical outcome parameter for success, but success can also be measured in terms of frequency of hypoglycaemic episodes and positive effects on vascular complications or quality of life[9]. Researchers found that islet transplantation often could not achieve long term insulin independence. Patients with this "partial graft function" have persistent insulin secretion from ß cells

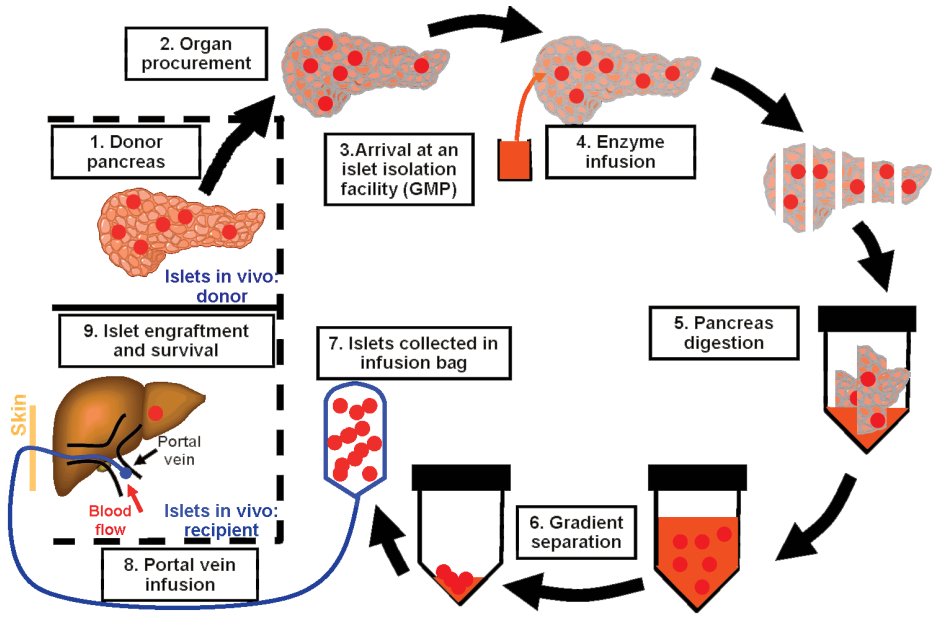

Fig 3 | The islet isolation and transplantation procedure. Islet isolation from a donor pancreas is laborious, time consuming, and costly. A donor pancreas (1) is allocated to a potential recipient on the waiting list, procured (2), and transported to an islet isolation facility (3), which adheres to good manufacturing practice guidelines (box 2). At the facility, enzyme is infused into the pancreatic duct (4) and the islets are separated from the exocrine pancreatic tissue by combined enzymatic and mechanic digestion (5), then purified by density gradient centrifugation (6). Reported numbers of isolated islets vary greatly; an estimated 300 000 to 600 000 islet equivalents (mathematical conversion of varying islet sizes to equal a standardised islet of 150 µm in diameter) can be isolated from one pancreas[44]. The actual number depends on the number of islets in the donor pancreas and the islet yield after isolation. Most centres culture the islets in incubators for several hours to several days to perform safety and viability tests and prepare the recipients. Shortly before transplantation the islets are collected in an infusion bag (7). Transplantation involves the infusion of pancreatic islets into the hepatic portal vein (8). Access to the portal vein is usually achieved by ultrasound guided percutaneous catheterisation under local anaesthesia. The islets are infused over 10-30 minutes and embolise the small branches of the portal vein. Patients usually stay in hospital for several days. The islets will engraft in the recipient liver (9) and begin to function

but require additional oral or subcutaneous antihyperglycaemic agents, such as insulin. A retrospective cohort study found that the hypoglycaemia score (measure of severity of hypoglycaemia) of 31 islet transplant recipients was significantly reduced from 5.29 (standard deviation 1.51) before transplantation to 1.35 (1.92) at an average 47 months after transplantation, indicating a substantial benefit even with partial graft failure and subsequent loss of insulin independence[10]. Partial graft function has been shown to be associated with reduced frequency and severity of hypoglycaemic episodes and increased quality of life[9]. Today, most clinicians regard an absence of severe hypoglycaemic episodes and return of hypoglycaemia awareness as indicators of successful islet transplantation.

WHAT RESULTS HAVE CLINICAL ISLET TRANSPLANTATION STUDIES SHOWN?

There are currently about 1000 recipients of islet transplantations worldwide. No randomised controlled trials have evaluated the effectiveness of the intervention. Small observational studies have been heterogeneous in their design. We review the best evidence from relatively large studies performed in established centres. Most studies report on patients with type 1 diabetes who had glycaemic lability, recurrent hypoglycaemia, and hypoglycaemia unawareness despite optimal self management. We focus on outcome parameters in terms of insulin independence and effects on vascular complications, quality of life, and patient survival.

ADDITIONAL EDUCATIONAL RESOURCES

Additional resources for healthcare professionals

Fiorina P, Shapiro AM, Ricordi C, Secchi A. The clinical impact of islet transplantation. *Am J Transplant* 2008;8:1990-7

Bretzel R, Jahr H, Eckhard M, Martin I, Winter D, Brendel M. Islet cell transplantation today. *Langenbecks Arch Surg* 2007;392:239-53

Low G, Hussein N, Owen RJT, Toso C, Patel VH, Bhargava R, et al. Role of imaging in clinical islet transplantation. *Radiographics* 2010;30:353-66

Collaborative Islet Transplant Registry (www.citregistry.org/) —Map of affiliated transplant centres and regular updates on all recipients registered

Lecture by L Fernandez of the University of Wisconsin on islet of Langerhans transplantation. http://videos.med.wisc.edu/videoInfo.php?videoid=1112

Animation on islet cell isolation. www.youtube.com/watch?v=aMNKu-ZVUls

European Association for the Study of Diabetes. Stem cells to cure diabetes: where do we stand? http://webcast.easd.org/Halban/index.htm

Additional resources for patients

Diabetes UK (www.diabetes.org.uk/Research/Islet_cell_transplantation/) —Comprehensive information on the islet transplantation procedure and eligibility criteria

National Institutes of Health (http://diabetes.niddk.nih.gov/dm/pubs/ pancreaticislet/) --More detailed information with links to USA based clinical trials

Juvenile Diabetes Research Foundation (www.jdrf.org.au/living-with-type-1-diabetes/what-is-type-1-diabetes) —Website on what type 1 diabetes is and how you can help further research in this area

Insulin independence

In 2000 a landmark case series reported on seven patients one year after islet transplantation. The seven recipients had remained insulin independent for an average of 11 months. The results of this small study were enthusiastically received[1;11]. It also became clear, however, that most patients needed two to three donor islet infusions to achieve insulin independence and that insulin independence was rarely sustained. Follow-up of a larger cohort of 65

Box 2 | Good manufacturing practice.

Good manufacturing practice is part of a quality system for the manufacturing and testing of foods, diagnostics, active drug ingredients, drug products, and medical devices. Islets of Langerhans, as a drug and biological product, are included in this quality system. In Europe, fewer than 15 islet isolation facilities currently generate islets for transplantation. Good manufacturing practice guidelines and enforcement are subject to country or continent specific legislation (see websites below).

World Health Organization (www.who.int/medicines/areas/quality_safety/quality_
assurance/production/en/)

European Union (http://ec.europa.eu/enterprise/sectors/pharmaceuticals/documents/
eudralex/index_en.htm)

United States (www.fda.gov/Food/
GuidanceComplianceRegulatoryInformation/
CurrentGoodManufacturingPracticesCGMPs/default.htm)

Canada (www.hc-sc.gc.ca/dhp-mps/compli-conform/gmp-bpf/index-eng.php)

Australia (www.tga.gov.au/docs/html/gmpcodau.htm)

patients reported in 2005 showed that insulin independence was present in about 69% at one year, 37% at two years, and 7.5% at five years. However, C peptide—a measure of insulin secretion (for every molecule of insulin one molecule of C peptide is released from ß cells)—was detected in 82% of subjects, indicating persistent but insufficient islet graft function at the end of this study[12]. More recently, in a cohort of 14 patients, about 64% were insulin independent and 83% had detectable C peptide at two years of follow-up[13]. The multicentre voluntary Collaborative Islet Transplant Registry (CITR) reported on 412 allograft recipients recruited from 1999 to 2008 with three year follow-up data for 257 islet transplant recipients[14]. At three years, about 27% of recipients were insulin independent, C peptide was detected in about 57%, and 16% of the patient data were missing[14]. Thus, long term partial graft function seems to continue and be expressed clinically by more stable glucose control and lower insulin requirements. Indicators of declining islet graft function in patients who have resumed insulin administration are worsening of glycaemic control, higher insulin demand, and a reduction in C peptide concentrations. Recent trials using a single islet infusion and new immunosuppressive protocols showed promising results at one year[15;16]. After one islet infusion all five patients treated with a belatacept based immunosuppressive regimen were insulin independent at one year[15].

Vascular complications

Islet transplantation is associated with improvement or stabilisation in microvascular complications (neuropathy, retinopathy, and nephropathy) and cardiovascular outcome

parameters[8;17;18]. An important clinical question, however, is whether it reduces microvascular complications more effectively than optimal glycaemic control achieved by subcutaneous insulin administration. Because no randomised controlled trials have been performed, we report the findings of one study of 42 patients that compared the effect of islet transplantation versus intensive medical treatment on microvascular complications using a one way crossover design[18]. This study found that islet transplantation improved glycated haemoglobin (6.6 (0.7) *v* 7.5 (0.9)), halted progression of retinopathy (0/51 *v* 10/82 eyes), and stabilised glomerular filtration rate compared with intensive medical treatment. In a prospective study of 44 patients with type 1 diabetes and previous kidney transplantation, islet transplantation performed in 24 patients improved kidney graft survival at six years compared with kidney transplantation alone (86% *v* 42% kidney graft survival, respectively)[8]. Improved cardiovascular function after islet transplantation was shown in the same patient group[17].

Quality of life

Several groups have studied the effect of islet transplantation on health related quality of life[19;20]. Recipients of islet transplants have indicated that stable glucose control and absence of hypoglycaemic episodes are the most beneficial outcomes of the procedure, providing a feeling of reliability and improved independence[21].

Patient survival

Whole pancreas transplantation has been shown to improve patient survival[22]. Because of the small number (about 1000) of patients who have undergone islet transplantation worldwide, the short length of follow-up, and the small size of individual studies, it is not yet known whether islet transplantation improves survi

ONGOING RESEARCH AND UNANSWERED QUESTIONS

- How can the islet yield be improved to decrease the number of donors needed for one successful transplant[39]?
- Identifying the best islet implantation site and technique that will result in an optimally functioning graft[29]
- How can biomaterials be used to create alternative transplantation sites?
- Which in vitro tests can best predict in vivo functioning of transplanted islets[40]?
- What alternative cell sources (such as embryonic stem cells or tissue specific progenitor cells) can be used to overcome the shortage of donor organs[41]?
- What immunosuppressive strategies are less toxic to ß cells?
- Can tolerance be induced by cellular immunotherapy, thereby making immuno-suppressants obsolete[42]?
- What are the key factors in long term islet allograft failure?
- How can islet mass be visualised and monitored[43]?
- How can long term islet function be improved?

WHAT AFFECTS OUTCOMES?

Box 3 and fig 4 list some of the factors that can lead to the loss of islets of Langerhans before, during, and after transplantation.

Box 3 | Factors that contribute to islet loss before, during, and after transplantation.

Factors affecting islet yield and quality

Donor characteristics
Organ procurement
Preservation and transportation
Isolation technique
Culture conditions

Factors contributing to loss of transplanted cell mass during and after transplantation

Immediate blood mediated inflammatory reaction
Recurrence of autoimmunity
Toxicity of immunosuppressive drugs
Allorejection
Glucotoxicity
Hepatic steatosis

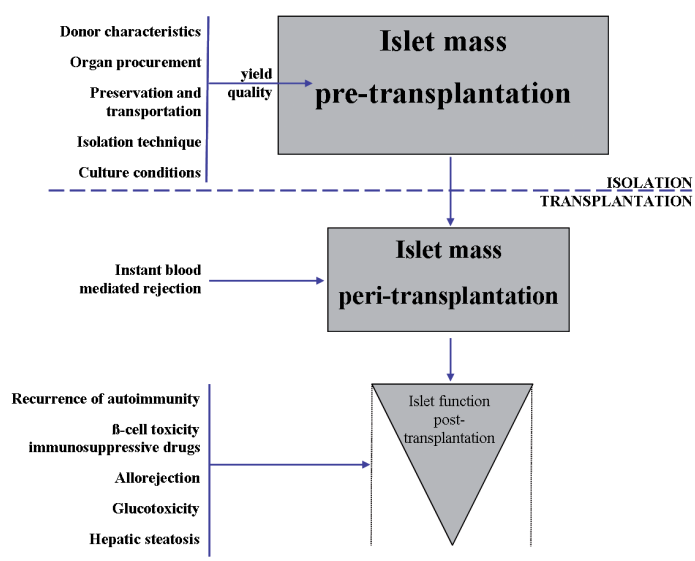

Fig 4 | Islet loss before, during, and after transplantation.

Pretransplantation and peritransplantation factors

Although glucose concentrations immediately normalise after successful whole pancreas transplantation, glucose lowering after islet transplantation is delayed. This is probably because an insufficient number of functional ß cells are transplanted. A single islet infusion—the islets of one donor—is often insufficient to establish normoglycaemia. Donor characteristics, the procurement of the donor pancreas, pancreas preservation during transportation, the islet isolation procedure used, and culture conditions have important effects on the number and quality of transplantable islets[23]. A substantial loss of islets is also thought to occur during transplantation[24], mainly because direct contact of islets with blood components in the hepatic portal system triggers an immediate blood mediated inflammatory reaction[25]. Thus, often an inadequate or marginally adequate islet mass reaches the liver tissue. Several measures can help avoid this loss of functional islet mass, such as administration of heparin during and after transplantation[26] and perioperative delivery of anti-inflammatory agents[27]. Still, many experts believe that the best way to improve the outcome of islet transplantation would be to prevent inflammatory reactions during and immediately after islet transplantation.

Post-transplantation factors

After infusion into the portal vein, the islets travel to the liver. Here they need to adjust to their new environment and also face adverse conditions. The islets are immediately exposed to drugs and nutrients, such as glucose, which are present in higher concentrations in the portal system than in the peripheral circulation, and which can negatively affect islet function. One of the obvious potential problems is acute rejection, for which immunosuppressive drugs are given. Unfortunately, some immunosuppressive drugs, such as calcineurin inhibitors and steroids, interfere with ß cell function[28]. Measures that can help to give the islets a favourable start include using immunosuppressive drugs that have little effect on glucose metabolism and strict glycaemic control to avoid glucotoxicity[26]. In addition, alternative implantation sites are being sought to avoid triggering the immediate blood mediated inflammatory reaction and the toxic drug levels found in the liver, and at the same time optimise vascularisation of the transplanted tissue[29]. Recently, islets have also been transplanted in human forearm muscle[30]. The omental pouch, bone marrow, and implants consisting of islets within a biomaterial structure (scaffolds), are other potential transplantation sites[29]. Islet revascularisation occurs within several weeks, but the intra-islet vascular network is less developed in islets transplanted into the liver than in eutopic pancreatic islets[31]. Thus, if not rejected early, the islet graft may not reach maximal efficacy with respect to glucose metabolism until one to three months after transplantation.

After one to three months islet efficacy becomes apparent, but on average only half of patients remain insulin independent at 15 months[9]. Chronic allograft rejection is a potential cause of long term graft failure[32]. Autoimmunity may also recur because islet recipients with positive T cell responses to autoantigens are more likely to lose full graft function[33]. Furthermore, the long term toxic effects of immunosuppressive drugs on ß cells are probably of considerable importance[28].

In patients who remain insulin independent after islet transplantation, a substantial portion of ß cell mass may already have been destroyed before glucose concentrations start to rise. The absence of methods to monitor ß cell mass, or alloimmune and autoimmune reactivity against ß cells, render the intrahepatic grafted islets a "black box." Whereas in whole organ transplantation, biopsies provide information on potential problems such as rejection, ischaemia, and immunosuppressive toxicity, it is difficult to biopsy the islets dispersed throughout the liver. Liver biopsies have been performed to evaluate transplanted islets by light microscopy[31]. However, this is an invasive procedure with low islet sampling rates and lack of reference values, which has limited value in clinical practice. Consequently, when islet function decreases and glucose concentrations rise over time there is little basis for intervention strategies other than re-evaluating the need for immunosuppressive drugs that negatively affect glucose metabolism and the use of glucose lowering agents. Therefore, current research is focused on increasing the functional ß cell mass before, during, and after transplantation and on improving the functional assessment of grafted islets[34].

WHAT ARE THE POTENTIAL COMPLICATIONS OF ISLET TRANSPLANTATION?

Complications can occur early (procedure related) or late (usually related to the use of immunosuppressives). Reports of early procedure related complications have come from different centres with a variety of expertise that have performed varying numbers of transplants. We try to give an indication of how often complications arise, how to monitor them, and how to try to prevent them.

Short term procedure related complications

Islet transplantation is a minimally invasive procedure compared with whole pancreas transplantation. Few detrimental procedure related complications exist. Hepatic bleeding during transhepatic portal vein catheterisation occurs in about 12% of infusions[12], but this has become less common with the use of fibrin sealant, Gelfoam pledgets, or coils to seal the catheter tract on withdrawal of the catheter[35]. Hepatic bleeding into the peritoneal cavity usually resolves spontaneously. Only rarely is surgery needed and no detrimental effect on graft survival has been reported. The infusion of foreign cell material into the portal system inevitably poses a risk for portal vein thrombosis. In an experienced centre this complication occurred in less than 4% of islet infusions[12]. Low dose heparin, given prophylactically during and after transplantation, limits the risk of portal vein thrombosis and carries an acceptable increased risk of bleeding. The liver parenchyma surrounding the new islets is temporarily damaged, but this is entirely reversible probably because of the excellent regenerative capacity of the liver. Resolution of the damage can be monitored by measuring liver enzyme concentrations after transplantation.

Long term complications

Similar to other transplants, long term complications are mostly related to the side effects of systemic immunosuppressive agents. Systemic immunosuppression increases the risk of infections and cancers, particularly virus related skin cancers and certain lymphoproliferative disorders. The most widely used agents in organ transplantation are calcineurin inhibitors. Unfortunately, these agents also have a nephrotoxic effect, which increases the risk of worsening renal function, especially in patients with diabetic nephropathy. The risk of complications can be reduced and their early management ensured by monitoring drug concentrations to prevent overdosing, using measures to prevent and recognise the development of infections, having a low threshold for starting antibiotics and antivirals in transplant recipients, and regularly checking for dermatological complications.

Organ transplantation can lead to the formation of anti-HLA antibodies. Recipients of islet transplants are usually exposed to a wide range of HLA antigens from multiple donors because over time they usually receive several islet infusions matched for ABO blood group only[36]. Although antibodies to donor derived HLA antigens are detected in only a minority of islet transplant recipients taking immunosuppressive drugs, patients taken off these drugs, either because of transplant failure or immunosuppressive related toxicity, show an increase in these antibodies[36]. This is important in patients who develop end stage diabetic nephropathy and require kidney transplantation because the presence of anti-HLA antibodies limits the chance of finding an acceptable donor kidney. Currently, we have no way to prevent the development of such antibodies.

WHAT SHOULD I TELL MY PATIENT WHO ASKS ABOUT THIS PROCEDURE?

Islet transplantation has been shown to be beneficial for a specific group of patients with type 1 diabetes who have severe glycaemic lability, recurrent hypoglycaemia, and hypoglycaemic unawareness, although lifelong use of immunosuppressive drugs is necessary. The lack of randomised control trials prevents a thorough comparison between this procedure and best medical practice (intensive insulin treatment) or pancreas transplantation. This lack of evidence has led to scepticism about the clinical value of this procedure among some diabetologists[37]. Currently the initial goal of long term insulin independence is achieved by only a small proportion of patients—an important message to communicate to potential recipients. However, the select group of patients treated with islet transplantation has shown improved glycaemic control, reduced frequency of hypoglycaemic episodes, and reduced rate of progression of vascular complications. Researchers now need to identify factors that will lead to better graft survival and function.

CONCLUSION

Although progression in the islet transplantation field is not as rapid as was envisaged[1], the pitfalls and difficulties of this procedure are now clearly identified, and advances in islet isolation, transplantation, and patient management are likely to improve the clinical outcome of islet transplantation in years to come.

NOTES

Cite this as: *BMJ* 2011;342:d217

FOOTNOTES

Contributors: Jan W Schoones, a trained librarian, helped compose our search strategy. We thank Bart L Hogewind, Bob A van Es, and Danielle Cohen for critical reading of the manuscript. IMB had the idea for the paper. HdK, EJdK, and IMB planned the content and wrote the first draft. JAB and TJR redrafted the manuscript. HdK, EJdK, and IMB produced the final manuscript. All authors are guarantors.
Funding: None received.
Competing interests: All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organisation for the submitted work; no financial relationships with any organisations that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influenced the submitted work.
Provenance and peer review: Not commissioned; externally peer reviewed

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6

RECONNECTING THE ISLET OF LANGERHANS: ENDOTHELIAL REVASCULARIZATION, LYMPHATIC VESSELS AND NEUROGENESIS AFTER ISLET TRANSPLANTATION

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Submitted for publication

ABSTRACT

Islet of Langerhans transplantation is a relatively new and promising type of ß-cell replacement therapy, which could be applied to patients with type 1 diabetes in various stages of their disease. Unfortunately, one-year graft functioning of the islet transplant has turned out to be rather disappointing worldwide. Islet transplantation in humans is still only performed within a research setting, and many studies are performed to investigate why short-term outcome is relatively poor. Much research has focused on islet isolation, storage, transplantation site, and rejection. In this review our focus is on the revascularization of both the blood vasculature and lymphatic system and on renervation after islet of Langerhans transplantation. These topics are currently investigated in small animal models, i.e. rats and mice, instead of humans. It is expected that our better understanding of these three issues in experimental models will greatly contribute to further improvement of human islet transplantation. In this review, we describe that targeting the induction of blood vessel revascularization and renervation will most likely prolong islet allograft survival. Preliminary findings on lymphangiogenesis after islet transplantation gives reason to assume that inhibition of this process would be most beneficial to islet transplant survival. Recent literature on experimental islet transplantation is used, and we elaborate on how developments could improve islet transplantation therapy for patients with type 1 diabetes.

INTRODUCTION

Diabetes mellitus is a worldwide disease affecting over 171 million people in 2000, with a projection to rise up to 366 million people in 2030. The majority of patients suffer from type 2 diabetes, but both types of diabetes are increasing. Approximately 10% of patients with diabetes suffer from type 1 diabetes (T1D), which is essentially caused by auto-immune destruction of the ß-cell population in the islets of Langerhans[1]. The incidence of type 1 diabetes has increased as well (3.4% annual increase; 1995-1999[2], while the reasons for this increase remain elusive. Exogenous insulin administration is the most frequently used therapy to control blood glucose levels in T1D. Unfortunately, this therapy cannot prevent blood glucose fluctuations associated with secondary complications such as retinopathy, neuropathy, nephropathy, and cardiovascular disease. Reinstituting endogenous insulin production by replacing the affected ß-cell population is the only means to halt or even reverse the disease[3]. Replacement of the ß-cell population can be achieved by either vascularized pancreas transplantation or islet of Langerhans transplantation. Vascularized pancreas transplantation is a major surgical intervention, most often performed simultaneously with a kidney transplantation, and thus in patients with end stage renal disease as a result of diabetic nephropathy and other secondary complications, with significant morbidity and peri-operative mortality[4;5]. Islet transplantation is a relatively new and promising treatment which could be applied in patients at various stages of their disease.

Islet transplantation is a minimally invasive procedure during which the islets are infused percutaneously via the portal vein under local anesthesia. Hospital stay does not exceed 2 days. Islet transplantation has the advantage of less donor material being discarded since many potentially 'good' organs which are unsuitable for whole organ transplantation because of donor and technical deficits[6], can be used for islet isolation and subsequent transplantation. Unfortunately, one-year graft function of transplanted islets has turned out to be rather disappointing worldwide. Since its first successful application in humans in 1978[7], it has become clear that after initial adequate graft functioning, practically all recipients become insulin dependent again with a 5-year graft survival of 6.5%[8]. Hypo-awareness is relatively well obtained.

Islet transplantation in humans is still only performed within a research setting, and many studies are being performed to investigate why short-term outcome is relatively poor[9]. Most of these studies use animal models to investigate various issues involved in islet transplantation outcome. In these models, the conditions under which islet transplantation takes place can be completely controlled. Most research is performed in small animals, i.e. rats and mice. In human islet transplantation, islets are infused into the hepatic portal vein where they will embolise small branches of the portal vein, while in rodent islet transplantation the preferential site for islet transplantation is underneath the kidney capsule. The site under the kidney capsule in rodents has the advantage of being easy to reach. The transplanted islets stay compartmentalized, and it is possible to confirm graft function by nephrectomizing the islet-bearing kidney. The first successful report on

rodent models with islets transplanted under the kidney capsule appeared in 1974, by Brown *et al*[10]. Ever since, experimental islet transplantation has generated important data that are relevant for the clinical care of patients with T1D in want of transplantation.

In the early years, research emphasis was on islet isolation, storage, transplantation site and rejection, the development of which made important contributions to refining models of islet transplantation[11]. More recently, attention was drawn to three important issues: 1) the vascularization and revascularization of the transplanted islets being of course essential for islet survival and function. 2) Lymphatic vessel formation emerged as a subtopic of the endothelial neogenesis theme. 3) Neuronal reconnection, which of the 3 topics discussed here is the most recently described item, is closely related to endocrine function. It is expected that our better understanding of these three issues in experimental models will greatly contribute to further improvement of human islet transplantation. In this review, we discuss the state-of-the-art literature of experimental islet transplantation, and elaborate on how its developments could improve islet transplantation therapy for patients with type 1 diabetes.

VASCULATURE: BLOOD VESSELS

In the whole pancreas, islets of Langerhans are intricately vascularized by small capillaries so that each specific islet cell is in direct contact with the vasculature[12], resembling a glomerulus-like structure[13]. Islets consist of at least 5 hormone producing cells: ß-cells which produce insulin and amylin; α-cells which produce glucagon; δ-cells which produce somatostatin; polypeptide cells which produce pancreatic polypeptide and ε-cells which produce ghrelin. Size of the islets and distribution of these cell types differ throughout the pancreas[14]. Vascularization is crucial for islet functionality, which is to keep metabolic homeostasis through hormone responses on blood glucose fluctuations. Islets receive 5-15% of the blood flow of the pancreas, although they compromise <1% of that organ. In addition, the islets have a higher oxygen tension and their vessels have a greater volume than vessels in the exocrine part of the pancreas[15-18]. The endothelium is essential as a barrier to keep autoreactive lymphocytes, ready to destruct the ß-cell, out of direct contact with the ß-cells.

The blood flow route through the islets under physiological conditions is incompletely understood. There are three theories on islet microcirculation, recently reviewed by Ballian and Brunicardi[16]. One suggests that blood flows first through the non ß-cell region before it enters the ß-cell region; the second suggests that the blood flows first through the ß-cells and than through the non-ß cell region. The third option is that the blood flows without a clear distinction between cell types. Most importantly, these different theories mainly reflect the lack of knowledge on normal islet physiology and on transplanted islet physiology. Recently, the assumption that a prototype islet even exists is under debate rendering the dispute on islet vascularization unresolved[14;19;20]. It is evident that we cannot begin to investigate what revascularization of the transplanted islets entails

for their function and survival, as long as we do not understand how the vascularization of islets in their entopic location is structured.

During islet isolation, the vasculature of the isolated islets is disconnected from the direct environment rendering the isolated islets ischemic. Subsequently, both in humans and in animal models, the islets are transplanted to an ectopic location where revascularization is not immediately established. Even when the transplanted islets are eventually revascularized, it is not inconceivable that the original level of blood flow, volume, and oxygen tension will never be reached. In fact, it was shown that the original islet architecture is altered upon transplantation, and although the orientation of the microvascular blood flow within the graft after revascularization appears the same [21] perfusion and oxygen tension are chronically impaired[15;22] most likely contributing to graft dysfunction and even failure. In a recent publication by Morini *et al*, it was shown that a microvascular network arises in the islet graft underneath the kidney capsule within 3-5 days[23]. Until that time, islets are dependent on diffusion of oxygen and nutrients from surrounding tissue and remain relatively hypoxic. Actual blood perfusion is established within 10-14 days. Remodeling of the morphology, through angiogenesis, continues up until 2-3 weeks post-transplantation when engraftment was considered stable[15;23]. Figure 1C depicts the endothelial lining of the microvasculature established 7 days after transplantation.

Recent publications on transplanted islets have shown that endothelial cells of both recipient and donor origin are involved in the revascularization process. The mixed origin of the vasculature imposes a situation of which the consequences in terms of rejection are not well known.

Much research has focused on the revascularization of the transplanted islet as an essential premise for its function. Optimizing the revascularization after islet transplantation is still the centre of attention of much research, concentrating on administration of angiogenic factors[24] or additional cell therapy[25]. Less emphasis has been put on other essential structure formations, such as lymph vessel formation and neuronal innervation. From embryology it is known that both lymph and neuronal patterns follow the blood vasculature[26;27]. The following paragraphs will discuss lymph and neuronal patterns in islet transplantation.

The knowledge gathered on the necessity of adequate and rapid revascularization of islets after islet transplantation might lead to therapeutic intervention strategies. For instance, erythropoietin (EPO) has been shown to reduce hypoxic and ischemic stress in kidneys of a non-human primate model[28]. The expression of the EPO receptor was shown in islets from several species, including human, and administration of EPO to an *in vitro* culture of isolated islets prolonged islet cell survival[29]. Experiments involving implants consisting of islets within a biomaterial structure provide means to apply pro-angiogenic growth factors (GF). When the matrix of the implant is supplemented with vascular endothelial GF and hepatocyte GF, the engrafted islets show enhanced vascularization compared to unsupplemented matrix islet recipients[30]. Concluding, although the exact mechanisms responsible for the revascularization of islets after transplantation are still partly unknown,

with knowledge gained over the last few years, therapeutic intervention has proven to be feasible and helpful, and may give the human islet transplant a better start and outcome.

VASCULATURE: LYMPH VESSELS

Entopically, islets of Langerhans lack lymphatic microvasculature although the adjacent exocrine tissue does have inter- and intra-lobular lymphatic vascularization[31]. Lymph function in the pancreas is comparable to that of other encapsulated organs such as the kidney and liver[31]. The lymph vasculature is of critical importance in the pancreas to drain excess proteolytic-enzyme-containing fluid from the interstitial space, which would otherwise damage the tissue[32].

During the autoimmune destruction of ß-cells leading to T1D, dendritic cell infiltration of the islets is facilitated by lymphatic vessels[33]. The way dendritic cells infiltrate islets was demonstrated in the well-defined spontaneously non-obese diabetic mouse model. This mechanism is 'nicely demonstrated' to exist in the spontaneously non-obese diabetic mouse model: When the exit of lymphocytes from tertiary pancreatic lymph nodes is blocked, the spontaneous non-obese diabetic mouse does not develop diabetes[34]. In a rodent model of human type 1 diabetes, the manifestation of diabetes could also be prevented by retention of activated immune cells in the lymph nodes[35]. These findings demonstrate that although lymphatic vessels are absent in the islets themselves, islets do have a close relationship to the lymphatic vasculature surrounding them. In view of the role of the lymph vasculature in the development of diabetes, an interesting notion would be that whereas it seems essential to stimulate the revascularization of blood vessels in the islets after transplantation, lymphatic vessel formation should be avoided around the islets.

The formation of lymph vessels in islet transplantation occurs as early as one week post-transplantation, with a small increase in abundance at 1 month and 9-12 months after transplantation[36]. Reported data differ on the exact location of the newly formed lymphatic vessels. At 7 days after transplantation, we have seen new lymph vessels formed at the boundaries of transplanted islets in close approximation to the kidney capsule, but not in the transplanted islets themselves (fig 1D). Other groups reported new lymph and blood vessel formation in close proximity to each other, lymph vessels in connective tissue between transplanted islets, and lymph vessels localized on the boundary between pancreatic tissue and kidney tissue[36]. Recently it was found that interfering with lymphatic function after islet transplantation in an allogeneic mouse model resulted in inhibition of lymphangiogenesis and prolonged islet allograft survival[37]. One of the three agents tested, FTY720, had previously been shown to be able to protect the islet allograft in an allogeneic mouse model from allo- and auto-immune destruction[38].

That lymphangiogenesis takes place after islet transplantation near the allograft[36] and that islet allograft survival can be prolonged by lymphangiogenesis inhibition[37;38] are the most important findings in lymphatic vessel formation in islet transplantation today. More is known about this subject in other transplanted organs. In an allogeneic transplantation

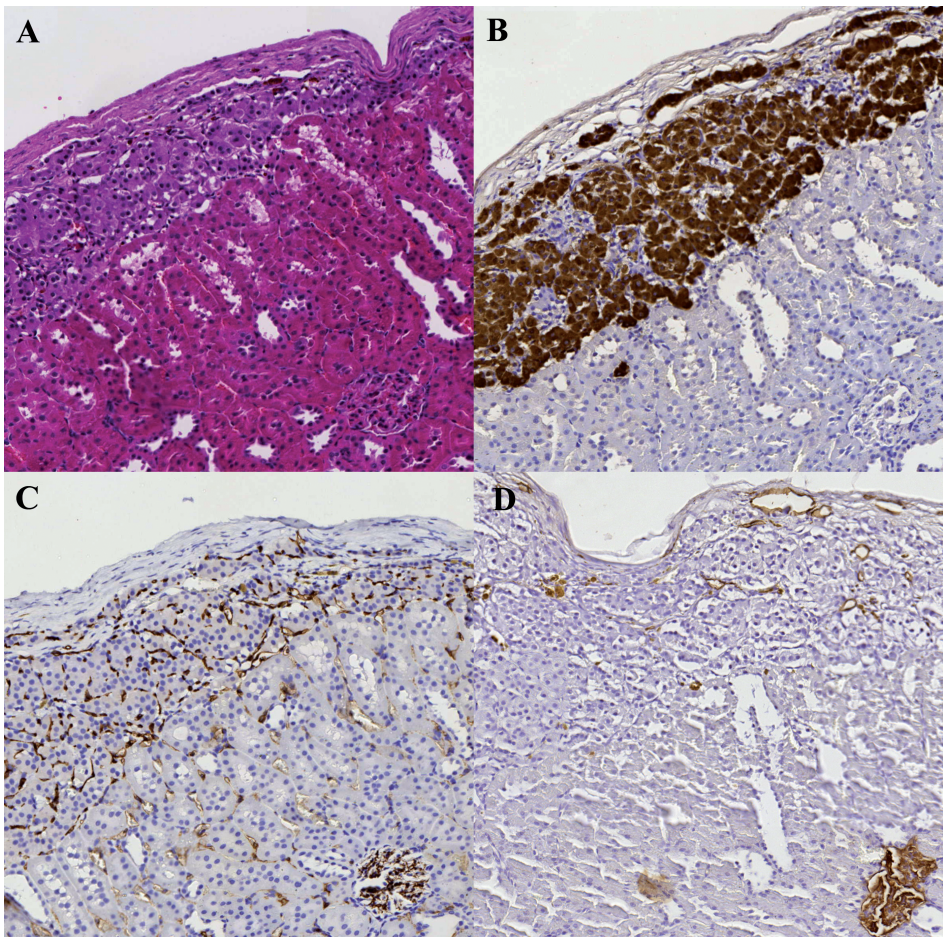

Fig 1 | Islet transplantation beneath kidney capsule of rodent model. (A) H&E, (B) insulin, (C) endothelial vessel staining (JG12), and (D) podoplanin.

setting, lymph vessel formation is considered to be of importance to facilitate drainage of antigen presenting cells to regional lymph nodes[39], which could be a driving force behind cellular rejection. However, in sequential protocol kidney biopsies taken at 6, 12, and 26 weeks after transplantation it was shown that lymphatic vessel formation within inflammatory infiltrates resulted in better graft function at one year, compared to the absence of lymphatic vessel formation at inflammatory sites[40]. Tertiary lymphatic structure formation has been described in transplanted kidneys with chronic allograft nephropathy, resembling secondary lymph nodes[41]. Tertiary intra-graft lymphoid organ formation appears to be driven by neurons which will be discussed below. The biological function of these tertiary lymphatic structures is supposed to be detrimental to the graft as these structures might lead to misguided immune responses whereby auto-antibodies are formed.

Perhaps the formation of tertiary lymph nodes is neither detrimental nor beneficial, but relies on the balance of both functions and is merely representative of chronic rejection[42-44].

It seems evident that in solid organ transplantation, research on new lymphatic vessel formation and tertiary lymphatic structures has only just begun, and the role of lymphatic vessels in terms of being beneficial or detrimental is unclear. At this point, it is known that new lymphatic vessel formation in islet transplantation takes place. Furthermore, with studies targeting lymphangiogenesis in islet transplantation in an allogeneic islet transplantation mouse model[37;38], clues on possible effective therapeutic interventions are gathered. The agents tested in mouse models should be transferred to larger animal models, where the islets are infused via the portal vein into the liver, to verify their potential in the human islet transplantation setting.

NEURONAL NETWORKS

Islets are richly innervated and have both sympathetic and parasympathetic nerves[45], but are mainly supplied with extrinsic nerves via the splanchnic and vagus nerves[46]. Several neuropeptides (vasoactive intestinal polypeptide, neuropeptide Y, calcitonin gene-related peptide, substance P) and classic neurotransmitters (noradrenaline) are known to have an influence on ß-cell insulin secretion. In type 1 diabetes onset, the auto-immune destruction of sensory afferent neurons promotes islet inflammation through altered glucose homeostasis and ß-cell stress[47]. In addition, from research in non-obese diabetic mice it is proposed that neurons and Schwann cells surrounding ß-cells within the islet are destroyed before the ß-cells themselves[48;49]. This emphasizes the essential role of neurogenesis after transplantation for a healthy and functional islet allograft. The upregulation of tissue factor (TF) in isolated islets known to cause the instant, blood-mediated, inflammatory rejection is also driven by neurons. It was established that the induction of brain death in combination with the warm ischemia time necessary to isolate islets, causes the expression of TF in isolated rat pancreatic islets[50].

The neurotrophic factors guiding neuronal in-growth are produced by the endocrine part of the pancreas and insulin could well be one of them[51]. It is feasible that due to islet denervation upon isolation and transplantation to an ectopic site, the allograft's function is impaired. Therefore, renervation might be another crucial area of interest, which few researchers seem to address even though it seems essential for normal ß-cell functioning. Several publications[52-54], imply that the innervation pattern in transplanted islets is altered, and that this is related both to factors within the transplanted islet, as well as to the environment of the transplantation site. This altered innervation pattern may affect the ß-cells' capacity for metabolic control. As a possible means to improve innervation of the islet allograft after transplantation, one study has co-transplanted neural crest stem cells with the islet transplant[55]. The co-transplanted neural crest cells interact with the islets and their addition results in improved islet functionality [55]. Whether it is the renervation or release of growth factors from these neural cells that induces the improved islet function is unknown.

After isolation of islets, nerve fibers are still present although completely separated from their environment. Already at two weeks after transplantation the first nerve fibers are observed which increase in number considerably between 26 and 51 weeks after transplantation. It has been proposed that this fiber in-growth occurs by accompanying the blood vessels revascularizing the graft[52;56;57].

It is suggested that adequate islet innervation is essential for normal islet cell functioning[58]. Still, the exact interactions between the endocrine part of the pancreas and the autonomic nerve system need to be established. From initial studies there is reason to assume that enhancing renervation, in this case via co-infusion of neural crest stem cells, can enhance islet function *in vivo*[55]. Therefore, targeting of the nervous system to improve islet transplantation outcome is likely to be beneficial.

CONCLUDING REMARKS

Transplantation of islets of Langerhans is a very promising cell therapy for an ever enlarging group of type 1 diabetes patients. Many areas of interest are studied as improvement is necessary to make it a success. Both the isolation and the transplantation of the islets pose many challenges, and the factors essential for advancement are still unclear. In this review we have shown that all processes involved in graft adaptation by the host through neuronal reconnection, lymph and blood vessel vascularization (figure 2) are intertwined and therefore the analyses of merely one cannot predict the outcome of islet transplantation.

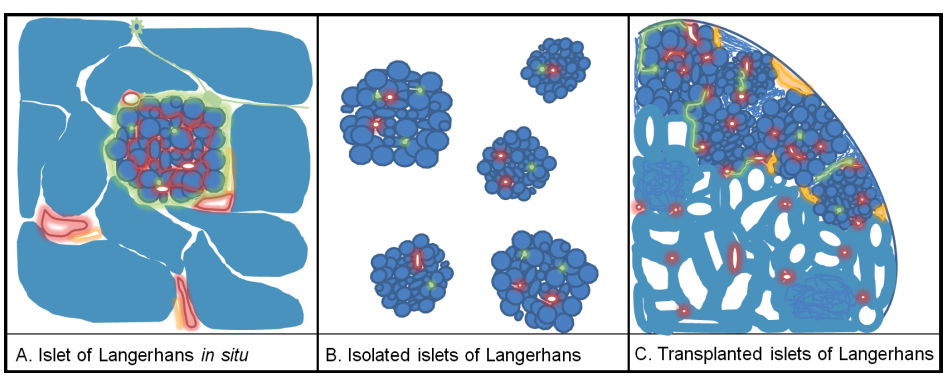

Fig 2 | Schematic depiction of islets of Langerhans in situ (A), after isolation (B), and after transplantation underneath the kidney capsule (C). Blood vessels (red), lymphatic vessels (yellow), and neurons (green) in all stages of islet of Langerhans transplantation.

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7

ACCELERATED ANTIBODY-MEDIATED GRAFT LOSS OF RODENT PANCREATIC ISLETS AFTER PRE-TREATMENT WITH DEXAMETHASONE-TREATED IMMATURE DONOR DENDRITIC CELLS

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Transplantation, accepted for publication after revision

ABSTRACT

Background

Allogeneic islets of Langerhans transplantation is hampered in its success as a curative treatment for type 1 diabetes by the absence of potent, specific, and non-toxic immunosuppressive drugs. Here, we assessed whether donor bone-marrow-derived, dexamethasone-treated dendritic cells (dexDCs), could prolong islet allograft survival in a full MHC mismatch rat model.

Methods

Rodent allogeneic islet transplantation was performed from DA rats to Lewis rats and vice versa. Permanently immature DCs were generated from the bone marrow of DA and Lewis rats by treatment with dexamethasone. Animals were either vehicle or donor dexDCs pre-treated. Serum was used to monitor glucose, C-peptide, and allo-reactive antibodies.

Results

The transplantation of DA islets into Lewis recipients showed direct graft failure with reduced numbers of ß-cells when rats were pre-treated with donor dexDCs. In the reverse model (Lewis islets into DA recipients), dexDC-treated DA recipients even showed a significantly accelerated rejection of Lewis islets. Immunohistochemical analysis of allograft tissue of dexDC-treated recipients showed a predominant NK cell infiltration and a presence of antibody reactivity in the absence of complement deposition. Allo-reactive antibodies were solely found in dexDC-treated recipients.

Conclusion

Our study shows that pre-treatment with donor-derived dexDCs induces an antibody-mediated rejection in this islet transplantation rodent model.

INTRODUCTION

Transplantation of islets of Langerhans is a promising cure for patients with type 1 diabetes. Thus far it is only being offered as a clinical research procedure[1] with a relatively good short term survival (1 year 69% insulin independence) but with a rapid decline in graft function overtime (5 year 7.5% insulin independence)[2]. Reasons for this loss of function include allograft rejection and complications caused by immunosuppressive therapy. Current immunosuppressive regimens are toxic to ß-cells, and influence insulin transcription, translation, synthesis, and secretion[3]. To inhibit rejection in a non-toxic way, alternative methods need to be developed, of which cell-based tolerance induction would be an attractive option.

DCs are bone marrow-derived, antigen-presenting cells that play an essential role in both innate and adaptive immunity. To prime the immune system and stimulate T-cells, DCs must undergo maturation, which can occur through a variety of signaling pathways after recognizing microbial and viral pathogens or inflammatory cytokines, or by CD40-CD40L binding[4]. However, when they remain immature, as in the steady state, DCs take up antigens and process and present peptides to T-cells in a tolerogenic manner[5]. Tolerogenic DCs have been shown to prolong allograft survival in various transplantation models[6-10]. Dexamethasone-pretreated, dendritic cells (dexDCs) have been reported to preserve a permanent immature phenotype and influence the immune system to create a tolerized environment through the induction of regulatory T-cells, which dampen the allo-immune response[11;12]. On the other hand, dexDCs have been proposed to lead to the processing and presentation of alloantigen by endogenous DCs, resulting in increased allo-immunity[13].

In islet transplantation, several immuno-modulating therapies have been shown to prolong allograft survival, such as hepatic DC progenitors[14], vitamin D3 with MMF[15], allopeptide-pulsed host DCs[16], intravenous infusion of Sertoli cells[17], and induction of donor chimerism through post-transplant donor-lymphocyte infusion[18]. However, the role of dexDCs in islet transplantation has not yet been investigated. In this study, donor bone marrow-derived, dexamethasone-treated dendritic cells were tested in a full MHC mismatch rodent model for their capacity to prolong islet allograft survival as well as their safety and efficacy.

MATERIALS AND METHODS

Animal models

Female 11-week-old Lewis (LEW/Crl, Germany) and Dark Agouti (DA) rats (DA/OlaHsd, Netherlands) were islet of Langerhans donors, male 8-week-old DA and Lewis rats were transplant recipients. Non-diabetic recipients were PBS injected i.v. and experimental recipients were rendered diabetic by a single freshly prepared streptozotocin (STZ, Sigma-Aldrich, Netherlands) i.v. injection (67.5 mg/kg in 0.91% w/v NaCl (pH 4.5)) under isoflurane anesthesia 2-3 days prior to transplantation. Diabetes was defined as 2 days of blood glucose levels >20 mmol/dl. All rats were housed under standard conditions and principles of laboratory animal care were followed in accordance with the animal ethical committee of the LUMC.

Generation of rat dendritic cells

BM was derived from both DA and Lewis tibias and femurs. DexDCs were generated from BM as previously described[12]. Briefly, BM was cultured at a density of $1.5*10^6$ cells/well in 3 ml of RPMI1640 (Invitrogen, Netherlands) containing 10% heat-inactivated fetal calf serum (FCS; BioWhittaker, Belgium), penicillin/streptomycin/fungizone (Gibco), ß-mercaptoethanol (50 M, Merck, Germany), L-glutamine (2 mM, Gibco), rat GM-CSF (2 ng/ml, Invitrogen), rat IL-4 (5 ng/ml, Invitrogen), and human Flt3L (50 ng/ml, kindly provided by Amgen). At days 2 and 4, medium was replaced by fresh medium containing the cytokines. For the generation of dexDCs, 10^{-6} M dexamethasone (Pharmacy LUMC, Netherlands) was added to the culture on day 4. On day 7, non-adherent and semi-adherent cells were harvested. DexDCs harvested and generated via this protocol were characterized previously[12].

Islet isolation

Under isoflurane anesthesia, the abdomen was opened and the rat was perfused with cold PBS via the descending aorta after clamping of the thoracic aorta, relieving pressure by opening the posterior vena cava. The common bile and pancreatic duct were clamped off at the duodenum, the common bile duct was cannulated, and 8 ml of cold Liberase RI or TL (Roche Diagnostics, Germany) in RPMI1640 (Sigma-Aldrich, Netherlands) was infused. The extended pancreas was excised and stored on ice in 2 ml Liberase RI or TL in RPMI1640, until all donor pancreata were collected. Animals were sacrificed in the process; femurs and tibia were used to collect BM.

All pancreata were simultaneously incubated at 37°C for 17 min, after which digestion was stopped with cold RPMI1640 containing 10% FCS, 100 mg/ml penicillin/streptomycin. Islets were separated by density-gradient centrifugation with 1.077 g/ml Ficoll-amidotrizoate (Pharmacy LUMC, Netherlands). Isolated allogeneic islets (800-1100 per recipient) were transplanted on the day of isolation.

Islet transplantation

Male Lewis or DA recipients were injected i.v. with PBS (vehicle) or 5×10^6 donor-derived dexDCs. Five days later, recipients were either rendered diabetic with STZ or mock injected with PBS. Two days after diabetes induction, islets of Langerhans were transplanted underneath the kidney capsule or a sham operation with vehicle was performed. All procedures took place under isoflurane anesthesia and during islet transplantation 0.01 mg/kg buprenorphine-hydrochloride (Temgesic, Schering-Plough, UK) was injected s.c. Prior to transplantation, the recipient received a s.c. injection of 1-1.5 U insulin (Insulatard, Novo Nordisk, Denmark). Transplantations were deemed successful when blood glucose levels dropped <11 mmol/dl and were deemed failures when glucose levels raised >14 mmol/dl. On days 2 or 7 days or more after transplantation, the recipients were sacrificed. First serum was collected, then the animal was perfused with PBS and subsequently kidney and pancreas were harvested.

Tissue analysis

For the Lewis to DA transplantation, each islet-bearing kidney was mounted on Tissue-Tek (Sakura, Netherlands) and snap-frozen in -80°C. All other organs were split in half and one half was snap frozen, while the other half was fixed in 4% buffered formalin for 24 h and paraffin-embedded, for (immuno-) histochemical analyses. Frozen samples were cryosectioned at 3-µm and stored at -20°C until use. For the DA to Lewis transplantation, all tissues were directly fixed and paraffin-embedded.

Immunohistochemistry

Frozen islets containing consecutive kidney sections were fixed for 10 min in acetone and then incubated at RT with the following primary mouse monoclonal antibodies: R73γ1 (α/ß TCR, 1:500, overnight[32]), ED1 (CD68, 1:5, overnight[33]), NK3.2.3 (NKR-P1, 1:120, overnight[34]), OX1 (CD45, 1:200, 1 h[35]), PL1 (platelet marker, 1:1, overnight[36]) OX6 (MHC RT1-B class II, 1:50, 2 h[37])) diluted in 1% BSA/PBS. Secondary antibodies (either horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG or IgG1) were diluted in 1% normal rat serum/ 1% BSA/PBS at 1:100 and, after an 15 min incubation, were applied to the sections for 60 min. HRP was visualized with 3-3'-diaminobenzidine (DAB). Then, the sections were dehydrated and prepared for light microscopic analyses.

Insulin staining was performed on formalin-fixed frozen sections. Frozen sections were fixed for 2 h in 4% phosphate-buffered paraformaldehyde and incubated at RT with rabbit anti-insulin antibody H-86 (1:100; overnight; Santa Cruz, Germany) in 1% BSA/PBS. Antibody binding was visualized with REAL™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (DakoCytomation, Denmark).

Indirect immunofluorescence (IIF)

To visualize donor-specific antibody formation upon Lewis-derived dexDC pre-treatment of DA recipients, serum of recipients collected at the termination of the experiment was incubated on frozen DA and Lewis pancreas control sections[38]. Serum diluted 1:10 in PBS was applied to acetone-fixed pancreas sections for 30 min at 37°C. Antibody binding was visualized by 1:200 FITC-conjugated rabbit anti-rat IgG (H+L) (KPL, USA) for 30 min at 37°C. Subsequently, the same slide was stained for OX6 as described above with the following changes. The secondary antibody used was Alexa Fluor 546-conjugated goat anti-mouse IgG1(γ1) (Invitrogen, Netherlands), and slides mounted with DAPI containing Vectashield (Vector Laboratories, USA) until further analysis by fluorescence microscopy.

Flow cytometry

BM-derived DCs from DA and Lewis rats were cultured as described above[12] and used for FACS analysis. Serial dilutions of serum from PBS- or dexDC-treated DA recipients were diluted in FACS buffer (PBS, 1% BSA, 0.02% Sodium azide) and incubated with the cells for 2 h. After washing, cells were incubated with 1:150 diluted goat anti-rat Ig-PE (BD Biosciences) for 1 h, washed and analyzed by flow cytometry (FACScalibur; BD Biosciences).

Statistical analyses

Graphs and analyses were computed with the use of GraphPad Prism for Windows, version 5.03 (GraphPad Software Inc., USA). Survival analyses were assessed by a log-rank (Mantel-Cox) test. Data are expressed as mean ± SD.

RESULTS

DA islets transplanted to diabetic Lewis recipients provoke rapid, immediate rejection irrespective of whether recipients were vehicle- or dexDC-treated

DA-derived islets of Langerhans were transplanted into diabetic Lewis recipients. These strains have been described as a high-responder allogeneic combination[19;20]. An immediate failure of the allograft was observed by blood glucose monitoring when no immunosuppression was administered. With an immunosuppression of 15 mg/kg/day cyclosporine A (CsA), rejection could be halted, but rejection occurred when CsA therapy was stopped (data not shown (DNS)).

Subsequently, the effectiveness of dexDC therapy was tested (Fig. 1A). DexDCs have been shown to produce IL-10 and completely lack IL-12 production, resulting in a reduced capacity to stimulate allogeneic T-cells *in vitro* and the capacity to induce T-cell hyporesponsiveness *in vivo*[21]. The blood glucose measurements of both groups showed the same results: an immediate failure of the allograft, even when treated with CsA. After transplantation, a short, transient drop in blood glucose levels was observed until day 5, which is consistent with the duration of CsA therapy, after which the blood glucose levels rise again (Fig. 1A). However, while in both PBS- and dexDC-treated recipients, no functioning graft was observed, grafted DA islets underneath the kidney capsule of Lewis recipients consistently showed residual insulin staining in the PBS-treated recipients (Fig. 2AB), but this was not observed in the dexDC-treated recipients (Fig. 2CD).

Pre-treatment with donor-derived dexDC abbreviates allograft survival in a model of Lewis islets transplanted to diabetic DA recipients

To gain a better idea of when immune regulation of the allogeneic islet transplantation was reversed, we transplanted Lewis islets into low-responder diabetic DA recipients[19;22]. In this model, an acceptance of the graft was observed for approximately 5 days, without CsA therapy (DNS).

Pre-treatment of diabetic DA recipients with donor (Lewis)-derived dexDCs resulted in accelerated graft loss (Fig. 1B). The PBS-treated recipients showed stable graft function for 5 days, after which rejection-induced graft loss began. After transplantation, the dexDC-treated recipients exhibited significantly increased levels of blood glucose above baseline (21 mmol/l compared with 11 mmol/l) while the PBS-treated recipients did not. Serum analyses at 7 days or more after transplantation showed a marked decrease in C-peptide in the dexDC-treated compared with the PBS-treated rats (234±46 pmol/l versus 301±83 pmol/l, respectively, p=0.069, normal rat C-peptide levels 450-900

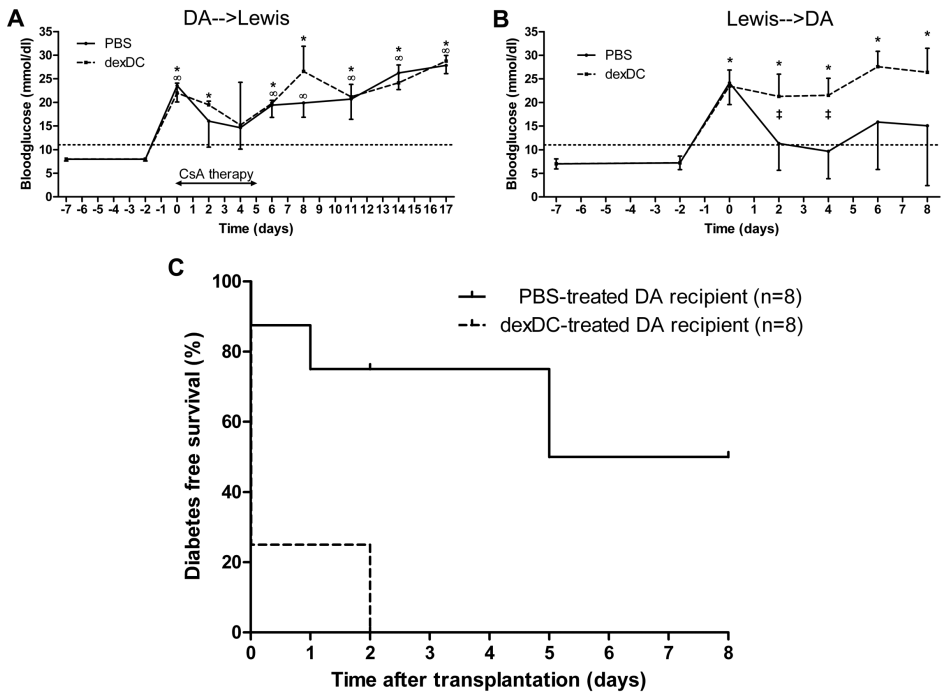

Fig 1 | Effect of donor-derived dexamethasone-treated dendritic cell therapy on islet allograft survival in DA to Lewis rats and Lewis to DA rats transplantation. (A) Blood glucose measurements of PBS-treated (black line, n=4) or dexDC-treated (dashed line, n=4) Lewis recipient groups that received DA islets (mean ±SD). On day -7, PBS or 5-10*10^6 DA-derived dexDCs were injected; on day -2, diabetes was induced by streptozotocin administration; on day 0, the Lewis rat received the transplant; and on day 10 the islet-bearing kidney was nephrectomized and paraffin-embedded for immunohistochemical analysis. (B) Blood glucose of PBS-treated (black line, n=8) or dexDC-treated (dashed line, n=8) DA recipient groups that received Lewis islets (mean ±SD). On day -7, PBS or 5-10*10^6 Lewis-derived dexDCs were injected; on day -2, diabetes was induced by streptozotocin administration; on day 0, the DA rat received transplant; and on day 2 or 8 the islet-bearing kidney was excised and snap frozen for immunohistochemical analysis. Dashed horizontal line indicates cut off blood glucose value by which the transplantation was deemed successful. At the time point indicated with *, the dexDC-treated recipients significantly differed ($p<0.05$) from the cut off blood glucose value. At time points indicated with ∞, the PBS-treated recipients significantly differed ($p<0.05$) from the cut off blood glucose value. On day 2 and 4, ‡ indicates a significant difference ($p<0.05$) between PBS-treated and dexDC-treated recipients.(C) Diabetes-free survival of DA recipients with or without dexDC pre-treatment. Day count started when blood glucose levels dropped below 11 mmol/dl and transplants were deemed failed when levels rose over 14 mmol/dl. The black dashed line indicates Lewis islet donor → DA recipient with Lewis-dexDC pre-treatment (cases, n=8) and the black solid line indicates Lewis islet donor → DA recipient with PBS pre-treatment (controls, n=8).

pmol/l). Furthermore, immunohistochemical analysis of Lewis islet-bearing DA kidney sections of the dexDC-treated animals (Fig. 2GH) consistently showed lower insulin content compared with the PBS-treated animals (Fig. 2EF). This finding is in line with observations recorded in the reversed high-responder rodent strain model.

The survival curve shows that only the Lewis islet allografts in the PBS-treated DA recipients survived (Fig. 1C), which significantly differed from survival of the Lewis islet allografts in the dexDC-treated DA recipients (p=0.002). Survival of the dexDC-treated versus PBS-treated Lewis recipients of DA islets were not significantly different (DNS). Importantly, this accelerated allograft loss and reduction in insulin expression upon donor-derived dexDC treatment was already present when experiments were ended 2 days after transplantation.

Immunohistochemical analyses of the dexDC-treated rats showed a distinct NK cell graft infiltrate in the islet allograft

To assess the mechanism of accelerated rejection, frozen tissue sections from kidneys of DA rats that had received Lewis islet allografts were investigated 2 and 8 days after transplantation. Infiltrates were characterized on sequential slides by several immunohistochemical markers (Fig. 3), including ones for T-cells, natural killer (NK) cells, platelets, and macrophages. The most distinct difference in the infiltrates was the large NK cell (Fig. 2IK) and cytotoxic T-cell population in the dexDC-treated group at day 2. At 7 days after transplantation, the remains of the transplanted islets were almost indistinguishable in the dexDC-treated recipients, while in the PBS-treated rats, the infiltrate at the islet transplantation site was still prominently visible (Fig. 2JL).

No difference could be found when comparing PBS- and dexDC-treated recipients with direct immunofluorescence (IF) for C3 deposition (Fig. 2MO). As expected, within the kidney, C3 deposition was seen along the tubular basement membrane in half-moon shapes and at the capsule, functioning as an internal control. In between the kidney capsule and the cortex, at the actual islet transplantation site, there was no C3 deposition. Direct IF with IgG showed no prominent IgG deposition, in line with the absence of C3. However, when indirect IF was used to examine the binding of serum on normal donor and recipient-derived tissue, IgG deposition was seen. When dexDC-treated DA recipient serum was incubated on donor Lewis-derived pancreas tissue co-localization of MHC class II (red) and serum-derived IgG (green) was observed (Fig. 2P). This staining was donor-specific, as the same serum did not show co-localization on recipient-derived DA pancreas tissue (DNS). Serum derived from PBS-treated DA recipients did not show co-localization, either on Lewis (Fig. 2N) or DA-derived pancreas tissue (DNS).

Serum samples of dexDC-treated rats show markedly higher allo-reactive antibody reactivity

To further confirm and quantify the presence of allo-reactive antibodies, serum samples were taken at different time points, incubated with bone marrow (BM)-derived DCs of donor origin, and monitored by FACS analysis. Serum of PBS-treated DA rats that had

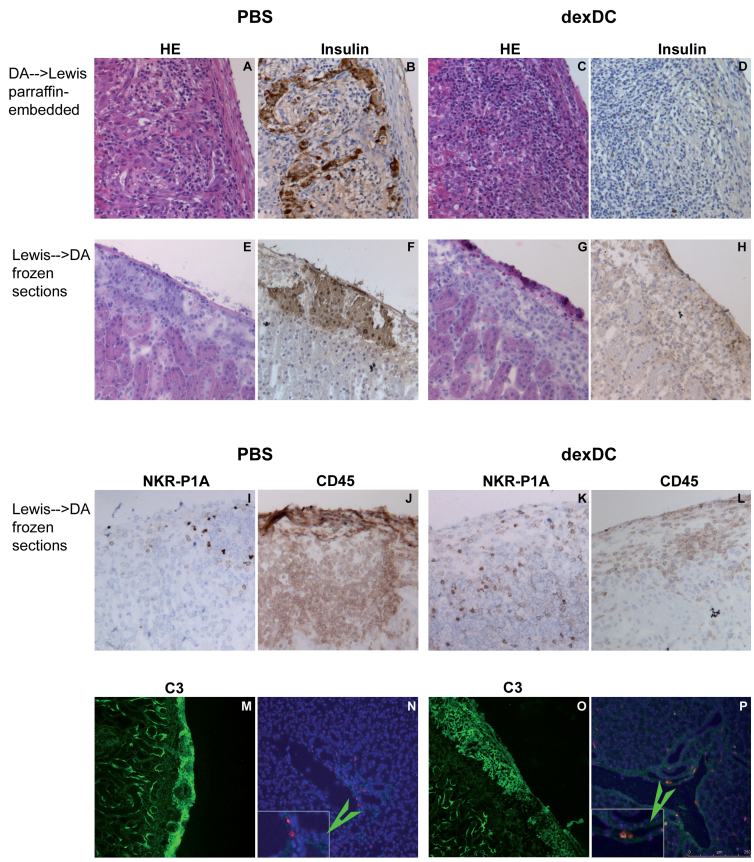

Fig 2 | (Immuno-)histochemical staining on paraffin-embedded islet-bearing kidney sections. (ABCD) Two Lewis recipients were transplanted on the same day, receiving 910 DA islets underneath their kidney capsules in combination with 4 days CsA therapy. Islet-bearing kidneys were procured 10 days after transplantation. (A) and (B) depicts a recipient treated with PBS 7 days prior to transplantation, while (C) and (D) shows a recipient treated with DA-derived dexDCs 7 days prior to transplantation. (Immuno-)histochemical staining on frozen islet-bearing kidney sections (EFGHIJKL). Two DA recipients were transplanted on the same day, receiving 1012 Lewis islets underneath their kidney capsules. Islet-bearing kidneys were procured 2 days after transplantation. (E), (F), and (I) depicts one DA recipient treated with PBS 7 days prior to transplantation. (G), (H), and (K) depicts one DA recipient treated with Lewis-derived dexDCs 7 days prior to transplantation. Two other DA recipients were transplanted on the same day, receiving 1100 DA islets underneath their kidney capsules. Islet-bearing kidneys were procured 7 days after transplantation. (J) was PBS-treated at day -7, (L) was dexDC-treated at day -7 (H&E (ACEG); insulin (BDFH); NKR-P1A (IK); CD45 (JL)). Direct immunofluorescence staining of C3 on frozen islet-bearing kidney sections (MO). Two DA recipients were transplanted on the same day, receiving 1055 Lewis islets underneath their kidney capsules. Kidneys were procured 2 days after transplantation. (M) was PBS-treated at day -7 and (O) was dexDC-treated at day -7. Indirect immunofluoresence, incubating dexDC-treated DA islet recipient serum procured at day 2, on a normal frozen Lewis pancreas section (green) in combination with an OX6 (major histocompatibility complex RT1-B class II) staining (red) and DAPI counterstain (blue) (NP). (N) was PBS-treated at day -7 and (P) was dexDC-treated at day -7. In the square, an enlargement of two single OX6 stained cells (N) and of OX6 and serum double stained cells (P) can be seen.

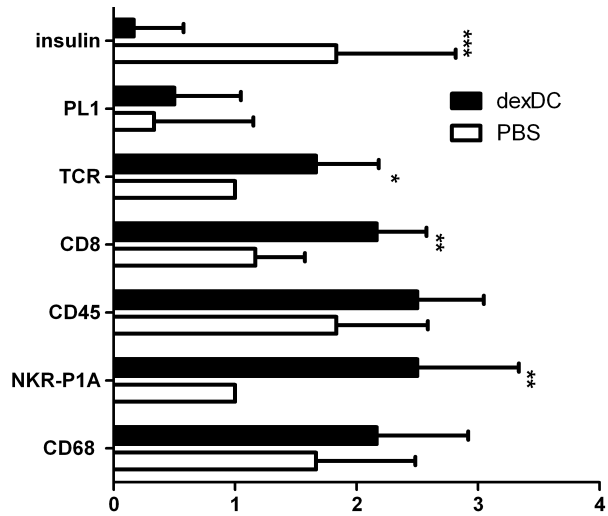

Fig 3 | Immunohistochemical analyses on frozen consecutive tissue sections of DA rat kidneys transplanted with Lewis islets at 2 days after transplantation. Black columns are dexDC pre-treated DA recipients (n=6) and white columns are PBS-treated DA recipients (n=6). All markers have been scored with a ranking system ranging from 0 (no staining) to 3 (extensive staining). Data are shown as mean ± SD, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. PL1, platelet marker; TCR, T-cell receptor, CD8, cytotoxic T-cell marker, CD45, leukocyte common antigen, NKR-P1A, natural killer cell receptor, and CD68, macrophage/monocyte marker. This style of representation was derived from other studies[39-41].

been transplanted with Lewis islets did not show a significant reactivity with Lewis DCs (Fig. 4A). In contrast, serum from DA rats, pre-treated with Lewis dexDCs and transplanted with Lewis islets, showed increased reactivity towards Lewis DCs compared with control DA serum (Fig. 4B). When analyzing different time-points, antibody reactivity was most pronounced 2 and 7 days after transplantation (Fig. 4C). However, already before islet transplantation, allo-reactive antibodies could be detected, indicating a priming of the humoral immune response by dexDC pre-treatment. Allo-reactive antibodies were not detectable in the recipients of Lewis islets without pre-treatment with Lewis dexDCs.

DISCUSSION

The preconditioning of islet transplant recipients with donor-derived dexamethasone-treated dendritic cells induces a hyper-acute, antibody-mediated rejection through the sensitization of the recipient for donor antigens. Blood glucose monitoring showed a significant difference in duration of graft acceptance between PBS- and dexDC-treated recipients. We have shown through several independent methods (FACS analysis using serum, and (in)direct immunofluorescence) that allo-specific antibodies were formed.

The use of dexDCs was derived from the safe DC vaccine trials for cancer, which proved to be efficacious and to have minimal side effects in some patients[23]. As testing of

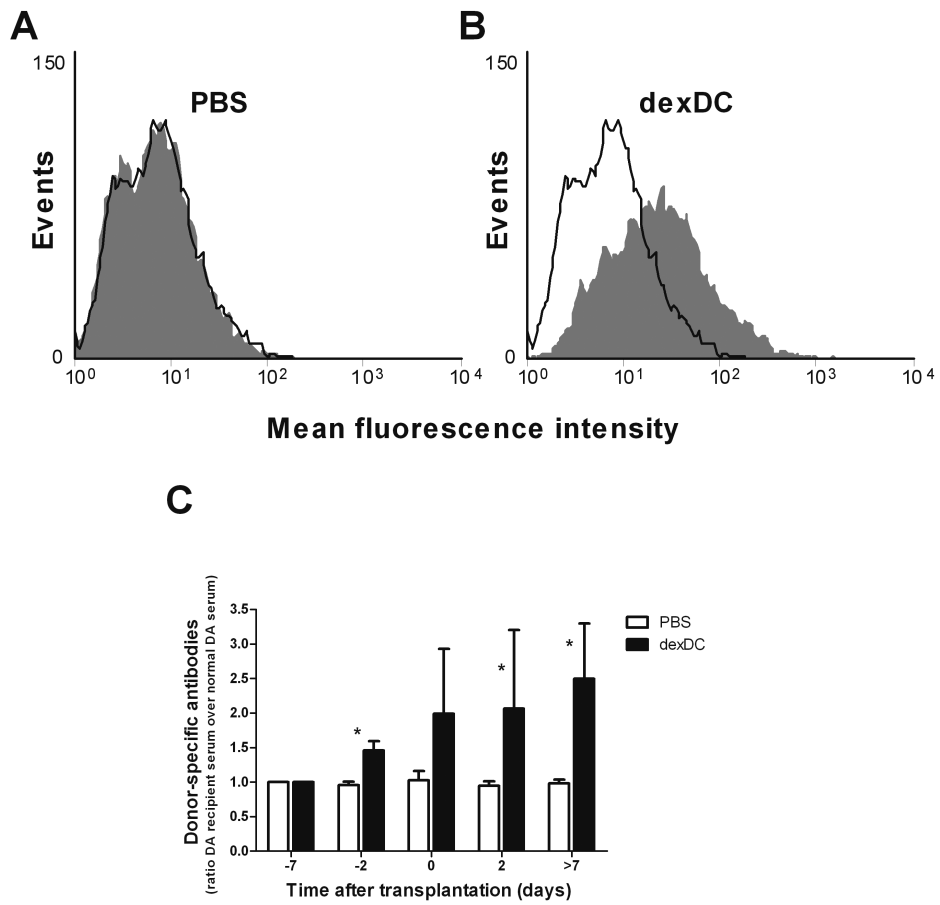

Fig 4 | Detection of donor-specific antibodies in DA recipients. (A,B) Representative FACS plot of Lewis BM-derived DCs incubated with (A) PBS-treated (control) DA serum at end of experiment, or (B) dexDC-treated (cases) DA serum at end of experiment (gray curve). Open curve is the control of the same cells incubated with normal DA serum. (C) The ratio of the mean fluorescence of Lewis-DC incubated with serum of DA recipients, divided by the mean fluorescence when incubated with normal DA serum (mean ± SD, * $p<0.05$). Comparison of PBS-treated (white bars) with dexDC-treated (black bars) at -7, -2, 0, 2, and >7 days after transplantation. All experiments with Lewis donors and DA recipients are included.

cellular therapy via DCs in human autoimmune disease is already underway, also the use of cellular therapy via DCs in transplantation and rejection becomes a realistic option[24]. In our model of allogeneic islet transplantation, strain-dependent reactions regarding rejection and tolerance induction were observed, as has been described in an allogeneic heart transplantation model[25]. Some have expressed their concerns that instead of inducing tolerance, dexDCs could lead to the processing and presentation of alloantigen by endogenous DCs, resulting in increased allo-immunity[13;26]. Recently, it was shown that allo-antibodies can facilitate not only antibody-mediated rejection (AbMR), but

also function as opsonins to enhance alloreactive T-cell priming[27]. The role of AbMR is becoming increasingly well-defined, and even low, "smoldering" levels of antibody are now thought to be responsible for the chronic deterioration observed in almost every allograft[28]. *In vitro* islets have been shown to be able to express HLA class II when stimulated[29] and in the clinical islet transplantation setting pre-formed HLA antibodies have been shown to reduce islet graft survival[30], allowing for the accelerated rejection observed in our sensitized islet transplantation model.

Similar dexDCs as the ones described in this study were used in other transplantation settings and have resulted in different outcomes. In an allogeneic full-mismatch kidney transplantation model using similar dexDC administration as in the present study, no allograft survival prolongation was found[21]. However, dexDCs did give rise to a donor-specific T-cell hypo-responsiveness. In both PBS- and dexDC-treated recipients, strong IgG antibody responses were found (unpublished data,[21]), but no indications of accelerated rejection were present. Alternatively, with a similar dexDC pre-treatment, prolongation of skin graft survival was reported, in which rejection was considered to have occurred when the tissue was fully necrotic, or the graft completely lost[8]. In the present study using an allogeneic islet transplantation model, the same dexDCs even accelerated graft loss. This occurred possibly because islet transplantation offers a unique situation in which the exterior of the cells come in direct contact with the blood of the recipient, while in whole organ transplantation, the endothelium forms a barrier. This most likely also contributes to the observation that in clinical islet transplantation recipients are prone to lose graft function through recurrence of auto-immunity while on immunosuppression, more so than recipients of vascularized pancreas transplants[31]. Therefore, islet recipients might be more prone to antibody-mediated effector mechanisms than pancreas transplant recipients, or recipients of other solid organs.

An accelerated rejection after dexDC induction therapy was shown in this study, which is most likely due to donor-specific AbMR. We have not been able to detect complement deposition (C3, C4 and C5b-9 immunohistochemical staining) at the site of transplantation, suggesting complement-independent effector mechanisms. Importantly, we showed a predominant NK cell infiltrate at the transplantation site in dexDC-treated recipients. This is different from the PBS-treated recipients, in which rejection was dominated by a T-cell infiltrate and occurred at a later time point. Our results strongly suggest that the rejection is complement-independent and that it most likely occurs through antibody-dependent cell-mediated cytotoxicity (ADCC). The speed of the rejection in particular favors a direct lysis of the allograft via ADCC in combination with the NK cell infiltrate observed. Human islet transplantation differs from the rodent transplantation model studied here in view of the streptozotocin-induced hyperglycemia investigated , and due to the fact that the recipient rats were not under the conditions that are required in human islet transplantation (humoral immunity, and immunosuppressive treatment). More research is necessary before cell-based therapy as an immunomodulating therapy can be considered in the human islet transplantation setting.

ACKNOWLEDGEMENTS

Part of these studies was funded by a collaboration with the Juvenile Diabetes Research Foundation international, Dutch Diabetes Research Foundation, and ZonMw (the Netherlands organization for health research and development); award number 2003.10.002. The manuscript was edited by American Journal Experts, managing editor: Bryce H., editor: Jillian A.

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8 SUMMARY AND DISCUSSION

In allograft transplantation, whether vascularized or cellular, the graft is potentially exposed to several adverse events, which include ischemia/reperfusion injury, acute rejection and immunosuppressive drug-related toxicity. Much scientific research is aimed at preventing graft damage caused by alloreactivity (i.e., acute rejection), which may eventually lead to an irreversible loss of function and/or premature graft failure. Two types of rejection have been defined: T-cell mediated and antibody-mediated rejection. Both types of rejection may occur either acutely or follow a more insidious and chronic course. The characteristic features and discriminating markers of these types of rejection are currently under investigation to identify the risk factors associated with inferior graft outcomes. To prevent rejection, immunosuppressive therapy is administered, and if rejection occurs, additional treatment is initiated and maintenance therapy can be adjusted accordingly. However, current immunosuppressive therapies may not always be adequate or specific enough to resolve the rejection process and may cause additional complications, including (opportunistic) infections. Therefore, alternative immunosuppressive strategies are being explored, with cellular immune modulation representing one potential approach. The envisioned outcomes of this research include the improvement of current short- and especially long-term graft survival rates through patient-tailored therapy regimens or, preferably, tolerance induction.

For kidney transplantation alone, the 5-year graft survival rates reported in the United States are 69.3% for deceased donor kidney transplantation and 81.4% for kidneys obtained from living donors[1]. With respect to simultaneous pancreas-kidney transplants (SPKT), the graft survival rates for the kidney and the pancreas are 78.6% and 73.4% at 5 years post-transplant, respectively. After islet transplantation, only 6.5% of patients remained insulin independent after 5 years[1;2]. Based on these numbers, it is obvious that further improvements are required for pancreas and especially islet transplantation. Rejection represents a serious threat to allograft survival. The work described in this thesis focuses on acute antibody-mediated rejection (AMR) in relation to allograft survival. AMR as a causative factor for the rejection of the kidney allograft was first described in 1993 by Feucht[3]. With increasing appreciation of its clinical relevance in kidney transplantation, the role of AMR in other transplant settings is beginning to be explored. New insights into AMR have fuelled research directed toward prolonging long-term survival of the renal allograft.

In this thesis, acute rejection after kidney, simultaneous pancreas and kidney, and islets of Langerhans transplantation was addressed. The focus of the thesis is on acute antibody-mediated rejection after pancreas transplantation and on a potential strategy using cellular immune modulation to prevent acute rejection. First, we retrospectively evaluated the relevance of diffuse C4d-positive peritubular capillary staining in a well-defined cohort with proven early acute rejections (Chapter 2). Second, the negative impact of AMR on pancreas graft survival was investigated, which proved to be significant (Chapter 3). We subsequently analyzed all SPKT patients at the Leiden University Medical Centre (LUMC) with early pancreas graft loss to examine the role of AMR in

early pancreas graft loss due to presumed thrombosis and/or acute rejection (Chapter 4). In Chapter 5, a clinical update is presented on islets of Langerhans transplantation, focusing on the alternative ß-cell replacement therapy that is currently employed in the LUMC (Chapter 5). Furthermore, we reviewed the role of both lymphatic- and blood-vessel vascularization and the role of neuronal reconnection after islet transplantation with data from our own rat islet transplantation models (Chapter 6). Finally, in a rodent allogeneic islet transplantation model, we attempted to induce tolerance by using donor-derived, dexamethasone-pretreated dendritic cells (dexDC) (Chapter 7). In the following paragraphs, these studies are placed in a broader perspective and future plans are discussed.

1. ANTIBODY-MEDIATED REJECTION

Antibody-mediated rejection is induced when donor-specific antibodies (DSA) are produced, which can bind to the endothelium of the allograft and cause classical pathway complement activation. This ability of the antibody proves its cytotoxic capacity, as complement activation leads to immune activation through the release of growth factors, cytokines, and chemokines and changes in adhesion molecule interactions and leads to lytic injury via the formation of the membrane attack complex (MAC), resulting in apoptosis. After antibody-induced complement activation, C4d is bound to the endothelium as a 'footprint' of the processes that have taken place. Histologically, AMR-induced damage can be represented by capillaritis (neutrophil and/or mononuclear cell margination in dilated capillaries), fibrinoid necrosis of arteries, and/or the presence of microthrombi, although none of these lesions are specific to AMR.

1.1 Antibody-mediated rejection in a historic perspective

AMR is a relatively new entity that was first discovered in the kidney transplantation setting. As outlined by Colvin, the occurrence of AMR after pancreas transplantation has been scarcely studied[4]. Figure 1 shows the distribution of publications on C4d per type of organ transplant according to the year of publication. The kidney was the first allograft in which AMR was described, and as represented in Figure 1, studies aimed at exploring the role of AMR in kidney transplantation have significantly increased over the years. Interest in AMR has now extended to other solid organ transplants, and it appears that the definition of AMR is not clear-cut in every type of allograft. For example, in small bowel transplantation, the relationship between C4d positivity and AMR is not evident[5]. In pancreas and heart transplantation, a first consensus agreement on the definition and criteria for the diagnosis of AMR has been established[6-8]. These agreements will be further discussed at upcoming Banff conferences on allograft pathology, and improvements are regularly made based on scientific advancements.

To diagnose AMR, most classifications require the presence of C4d-positive staining (>10% of specified capillaries), circulating DSA must be detected, and either graft dysfunction or certain defined histological lesions must be present in the organ. However, a biopsy is

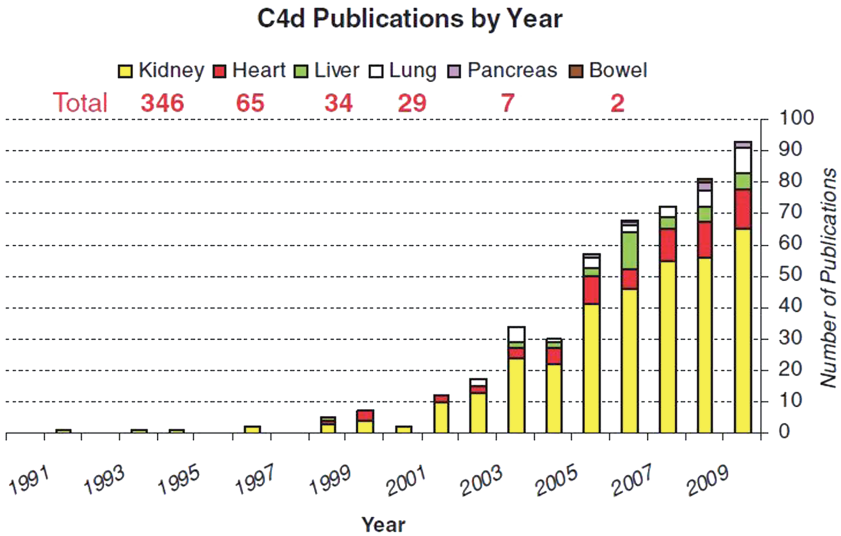

Fig 1 | Number of publications on C4d per transplantation organ per year[4]. Copyright (2011) Wiley. Used with permission (Colvin RB, Dimensions of antibody-mediated rejection, American Journal of Transplantation, John Wiley & Sons Inc.)

only a small sample of the organ at a specific moment in time. DSA have the advantage that they can be assessed systemically in allograft recipients; however, their relationship with injury at the tissue level may not always be clear. A schematic depiction of the proposed kinetics of DSA and C4d is provided in Figure 2. The depiction is speculative because the exact kinetics of the *in vivo* interactions of these two components is unknown[9]. Thus, a sampling error due to the timing of the biopsy or due to the timing of the serum sampling is entirely possible. Even so, DSA and C4d are still important tools for the diagnosis of AMR.

There are two reasons for searching for C4d depositions in the biopsy specimen instead of searching for the actual antibody binding to the endothelium. C4d is a general marker that is deposited irrespective of the specificity of the antibody, and as such, C4d deposition in the capillaries is a uniform marker for antibody-dependent complement activation. Of equal importance is the fact that C4d remains bound much longer and more potently than the actual antibody, providing a larger window of opportunity to find it as a hallmark of AMR. The length of time that C4d stays covalently bound to the place of activation after the first antibody priming is not known. Thus, when evaluating the results discussed in this thesis and in other studies on AMR, this basic lack of knowledge needs to be considered.

The studies described in Chapters 3 and 4 of this thesis contributed to our understanding of AMR in pancreas transplantation. In a multi-center international setting, we found that AMR, which was defined by the diffuse C4d deposition in interacinar capillaries in combination with *de novo* DSA in the recipient's blood, was associated with pancreas graft failure (Chapter 3). This study also provided the organ transplantation field with the first

histological correlates for AMR in the pancreas. We showed in an LUMC-based (1985-2010) case-control study of pancreas transplantation patients that some transplantectomy cases, which had been previously diagnosed as lost due to thrombosis, also showed signs of an AMR component (Chapter 4). Whether these grafts were in fact lost due to AMR or thrombosis remains to be determined. Prospective studies are necessary to establish the treatment of choice when *de novo* DSA and C4d deposition in the pancreas allograft are observed. Close monitoring of SPKT recipients for the occurrence of DSA to guide biopsies of both the kidney and pancreas allograft may prove to be the best strategy to improve early pancreas graft outcome. On the other hand, excluding specific HLA antigens in specific recipients may reduce the risk of AMR after pancreas transplantation to a level comparable to kidney transplantation.

In the study described in Chapter 4, all but one of the seven acute AMR cases (i.e., C4d deposition in combination with *de novo* DSA and morphological evidence of graft injury) with allograft loss were clinically categorized as loss due to rejection. The studies described in Chapters 3 and 4 showed that, in accordance with other studies[10-13], the transplanted pancreas and kidney from one donor may indeed show discordant rejection grades in simultaneously obtained graft biopsies. A prospective study may provide more insight in the divergence of alloreactivity at the tissue level of multiple organ transplants.

1.2 Chronic antibody-mediated rejection

Several types of AMR have been reported in the kidney: hyperacute, acute and chronic rejection. The use of the terms acute and chronic is not related to the time of occurrence after transplantation but is defined by the type of morphological changes due to the damage and/or remodeling present in the allograft[14]. In particular, chronic AMR is now considered relevant in late allograft failure, and AMR is not seen as an episode but as a process. Acute rejection may occur long after transplantation and can be induced by a decrease in immunosuppressive therapy, due to non-compliance, iatrogenic effects, and/or intercurrent malabsorption. Acute rejection is different from chronic rejection because in chronic rejection there is no clear option for acute intervention, and maintenance immunosuppressive therapy is continued mostly without major alterations to the regimen. Nonetheless, chronic damage will eventually lead to progressive graft dysfunction and premature graft loss.

Histologically chronic AMR is distinguished from acute AMR by the lack of inflammation markers such as neutrophils, edema, necrosis, and/or thrombosis. Regardless, DSA are considered to be an important contributing factor to chronic AMR; however, the contribution of the presence and levels of DSA to the development of either acute or chronic rejection remains unclear. Low levels of antibodies are thought not to provoke a direct reaction, while in the long term, these low levels may induce cumulative damage that can become fatal to the functional integrity of the transplanted organ. It was recently shown that the timing of *de novo* DSA development may have an impact on long-term graft survival[15]. In addition to DSA titers, the avidity of the specific antibody

or antibodies involved, their effector function, and endothelial resistance are all thought to play a role in the development of acute or chronic AMR.

The formation of antibodies has also been proposed as part of the tolerogenic process or in the adoption of an accommodative state in the recipient. *In vitro* studies have established that cells can be rendered insensitive to antibody-induced activation when exposed to prolonged suboptimal concentrations of this antibody[16;17]. The use of the words "accommodation" and "tolerance induction" for this phenomenon is still the subject of debate, but based on two studies (one in Europe and the other in the United States), it is clear that some patients will not reject their renal grafts while off immunosuppressive therapy, a condition known as operational tolerance[18;19]. The one remarkable alteration in the systemic immune cells found in both the European and the US studies is the up-regulation of specific B-cell markers. The significance of this finding needs further exploration, but it does seem to suggest a crucial role for antibodies in tolerance as well as rejection. Once the facilitating hallmarks of the renal allograft recipients who have acquired operational tolerance have been elucidated, it might be possible to wean other renal transplant recipients off immunosuppressive therapy in a patient-tailored manner[18].

Chronic AMR is currently a "hot topic" in organ transplant settings; however, in our pancreas transplantation studies (Chapters 3 and 4), we did not have the opportunity to study chronic AMR. The biopsy material was limited and tissue from the pancreas transplantectomies were mostly from graft losses within 1 year. Within that first year, one pancreas transplantectomy specimen showed chronic active AMR. Of the 66 transplanted grafts that were included as controls, with pancreas grafts that functioned well for at least 4 years, 8 patients later lost their grafts. Tissue specimens from 3 controls were retrieved from our archives, and only one showed the histological hallmarks of chronic AMR. Data on DSA in these cases were not available.

1.3 Relevance of C4d and donor-specific antibodies in antibody-mediated rejection

C4d without donor-specific antibodies

The marker C4d is used as a 'footprint' of antibody-mediated classical complement pathway activation. However, the sole occurrence of C4d is not specific enough to diagnose AMR, as further highlighted in the study described in Chapter 2. Circulating DSA are an essential part of a more reliable diagnosis of AMR, which has implications for clinical outcomes. A potential confounder, for instance, can be that C4d is deposited through AMR involving DSA other than the tested donor-specific anti-HLA antibodies. Therefore, one should be cautious in stating that AMR is absent in the case of C4d positivity without DSA. Some non-HLA antibodies that can be involved in AMR are not regularly tested, such as the major histocompatibility complex class I chain-related genes A and B (MICA, MICB), vimentin, agrin, glutathione-S-transferase T1 (GSTT-1), angiotensin type 1 receptor, and anti-minor HLA antibodies[20-24]. These antibodies may all play a role in AMR, and in all probability there will be many more antibodies against transplanted antigens still to be discovered.

Organ-specific reactions to antibody-mediated rejection

As has been shown in other studies[10;12;13], the findings described in Chapters 3 and 4 confirm that discordant, organ-specific rejection patterns can occur in the pancreas and kidney allografts of SPKT patients. When the duodenal patch attached to the pancreas allograft was studied (Chapter 4), only 69% of the cases showed consistency in the C4d staining of the duodenal patch, with C4d staining of the pancreas allograft. This gives rise to the speculation about the existence of organ-specific endothelial antibodies that are able to induce complement activation in specific organs from the same donor. The fact that these antibodies need to be able to bind and subsequently activate the complement system is established. However, the proteins that these organ-specific endothelial antibodies may bind to still need to be elucidated. Another explanation for the tissue-specific reaction to the *de novo* DSA as highlighted in Chapter 4, could be the tissue-specific endothelial expression of complement activation regulatory proteins such as the complement decay accelerating factor (DAF), the membrane cofactor protein (MCP), and CD59[25]. In addition, the finding of C4d deposition in the absence of DSA could be explained, for example, by the described ability of the renal allograft to absorb DSA from the circulation[26]. It has been observed clinically that following renal graft failure and transplantectomy, the antibody titers can rise immediately as the produced DSA are no longer absorbed. In these cases, AMR can be determined as the causative factor only after graft failure[26]. Another explanation for organ-specific AMR may be the presentation of tissue-specific peptides on HLA[27]. As more DSA epitopes are identified, the diagnosis of AMR may become even more complicated.

A new pre-transplantation screening method for antibodies is currently under investigation to gain insights into preformed endothelial antibodies and preformed donor-specific anti-HLA antibodies. The method involves pre-transplantation flow cytometric cross-matching of recipient serum on a panel of peripheral blood endothelial progenitor cells[28;29]. Unfortunately, this screening method is not able to discriminate between autoantibodies and alloantibodies because the target antigen expressed on the endothelial progenitor cells to which the antibodies bind is still undetermined. Another drawback might be that the characteristics of these blood-derived endothelial progenitor cells are different from fully differentiated endothelial cells. A possible alternative method would focus on the incubation of pre- and post-transplantation serum samples on both donor- and recipient-derived endothelial cells, with a read-out of either complement-induced cytotoxicity or flow-cytometry. Although such an approach would technically be feasible, the cost and complexity of the procedure makes it impractical for diagnostic purposes. Furthermore, the acquisition of endothelial cells from both donor and recipient would be a cumbersome exercise[30]. This method could provide an answer to the autoantibody or alloantibody characteristics of the *de novo* antibody, but the antigen it recognizes remains undetermined. A few commercially available endothelial antibody systems are currently on the market, and some validation studies have been undertaken. In a commercially available human umbilical vein endothelial cell (HUVEC) preparation and

antibody detection system by indirect immunofluorescence (Titerplane, EUROIMMUN, Germany), *de novo* anti-endothelial cell-antibodies were found after transplantation in the absence of C4d staining and in the absence of detectable common DSA tested for, but with histopathological signs of microvascular injury indicative of graft injury. Graft dysfunction was significantly more abundant in patients with detected *de novo* endothelial antibodies compared with recipients that remained negative for endothelial antibodies[31]. All in all, it appears likely that one of the standardized endothelial antibody detection systems might be added, in the near future, to the pre- and post-transplantation screening, in addition to the lymphocyte cytotoxic and solid-phase assays currently employed.

Donor-specific anti-HLA antibodies in antibody-mediated rejection

Donor-specific anti-HLA class I or II antibodies are found in 88-95% of patients with C4d deposition and graft dysfunction, while less than 10% of the C4d-negative patients have detectable DSA[32]. In a large kidney transplantation study at 1 year post-transplantation, only the sole occurrence of donor-specific anti-HLA class II antibodies was found to be detrimental to the graft, independent of C4ddeposition[33]A. A separate study on renal biopsies found that DSA determined at the time of the protocol biopsy were not indicative of graft function or survival. If DSA were present when a biopsy was taken when indicated, the graft function and survival were impaired compared with cases where no DSA were found at the time of indication biopsy[34].

The presence of DSA without C4d deposition was found in many studies, including ours as described in Chapters 3 and 4, and there are several explanations for this finding. First, all antibodies targeting HLA are picked up when using Luminex technology, independent of their ability to activate the complement system. Numerous DSA cannot induce a cytotoxic effect and as such are of no immediate threat to the allograft. Therefore, a cell-based cytotoxicity assay such as a complement-dependent cytotoxicity (CDC) assay is necessary to ensure the cytotoxic capacity of the detected donor-specific anti-HLA antibodies. To complicate things further, some antibodies are capable of inducing a cytotoxic effect without complement activation, a condition known as antibody-dependent cell-mediated cytotoxicity (ADCC)[35]. Recently, biomarkers at the mRNA level have been described to distinguish renal allograft rejection through ADCC by means of endothelial activation transcript signatures[36]. In Chapter 7, in all probability we unintentionally induced ADCC while attempting to apply cellular immune modulation toward a tolerogenic state in the rodent allogeneic islet transplantation model.

Several techniques can be used to detect anti-HLA antibodies, auto-antibodies, and other antibodies. There are solid-phase assays that use immobilized proteins and peptides as substrates, and there are cell-based assays using lymphocytes and, more recently, endothelial cells as substrates. The most important differences between these techniques lie in their specificity, sensitivity, and the costs involved per test[30]. The use of one technique over the other is based on the infrastructure per screening laboratory or country, the country-based regulations, and the information needed from the assay. Often, several steps

are taken to define DSA, starting with a relatively cheap, fast assay with low specificity and acceptable sensitivity to screen all samples. Based on the results gathered, the sample is further tested to determine the exact DSA and its capabilities. New techniques are still under development and the most optimal method has not yet been found[37].

Focal C4d staining and subclinical rejection

Focal C4d staining is defined as 10% to 50% of capillaries positively staining for C4d. In renal transplantation, the C4d staining is scored on peritubular capillaries, while in pancreas transplantation, C4d staining of the interacinar capillaries is scored. Especially in renal transplantation, the significance of focal C4d staining remains a topic for debate where some studies claim that it is predictive for AMR while others have not found a correlation with AMR and graft failure. For example, Kayler *et al* found that focal C4d staining in renal allograft biopsies was a harbinger of acute AMR[38;39]. Fior *et al* found that focal C4d staining was correlated with poorer graft survival[40]. Kedainis *et al* only found a trend towards poorer graft survival when focal C4d staining was present[41]. In a more recent study by Crary *et al,* immunoperoxidase C4d staining of over 10% of PTC was found to be predictive of graft loss[42].

As described in Chapter 2, the renal biopsies stained positive for C4d deposition in a small number of acute rejection episodes, but the C4d-positive episodes were of a more subclinical rejection type. Due to the small number of cases in our study, the power did not allow for the three categories (negative, focal, and diffuse C4d deposition) to be compared. We considered these C4d-positive cases subclinical because no difference between the C4d-positive and C4d-negative renal allograft recipients was observed, either in graft function or survival. Moreover, none of the biopsies showed the histological parameters of AMR. This is not a subclinical rejection per definition, described as AMR without graft dysfunction[43], but it is subclinical nevertheless. Some authors propose that the detection of C4d without any of the histological hallmarks of AMR may be an indication of graft injury that was easily repaired[44].

C4d-negative antibody-mediated rejection

C4d-negative AMR has recently been described by Sis *et al* on the basis of an mRNA expression study of renal biopsies[45]. Theoretically, the assumption that AMR can be present while the marker C4d has already lost its covalent binding with the endothelium is plausible (Figure 2), as is the assumption that AMR may occur through ADCC, inducing no complement activation while causing injury to the graft. The problem is how to distinguish these cases of AMR from the ones that have DSA without any damaging capacity. In other words, the question is which markers on the mRNA level show repair and which predict permanent damage. Sis *et al* claim to have found a way to answer this question through the use of endothelial activation (ENDAT) transcripts, which show a specific profile indicative of C4d-negative AMR. In the same study, other transcripts in renal biopsy-derived mRNA were assessed, and analyses revealed other cell cluster markers that were differentially expressed, such as the NK cell repertoire and the B-cell repertoire

markers[46;47]. Several groups are exploring the relevance of these and other molecular markers. In the United Kingdom, a validation study on specific ENDAT transcripts in renal transplantation has been initiated.

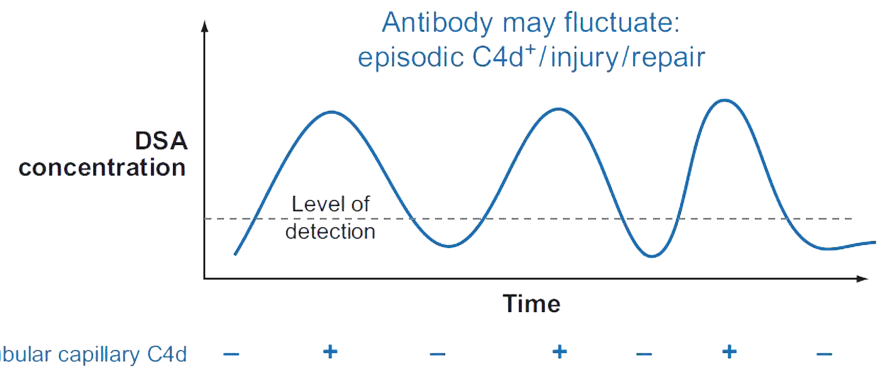

Fig 2 | Proposed kinetics of DSA in serum and C4d deposition in peritubular capillaries in kidney transplantation. Adapted from Cornell *et al*, 2008[9]. Annual review of pathology, Copyright 2008 by ANNUAL REVIEWS, INC. Reproduced with permission from ANNUAL REVIEWS, INC. in the Dissertation format via the Copyright Clearance Center.

1.4 Antibody-mediated rejection in pancreas transplants

The first reports on AMR in the pancreas were case studies. These case studies showed that AMR in pancreas transplantation was likely to represent a mechanism of graft rejection and that AMR had to be determined with a combination of C4d staining of the biopsy and donor-specific antibody determination. The first large cohort study in 2009 on 27 pancreas biopsies concluded that C4d deposition specifically in the interacinar capillaries is most likely associated with AMR, *de novo* DSA, and clinical outcome[48]. These results were followed by a study correlating C4d staining with several serum- and urine-derived markers in the rejection of the pancreas allograft[49]. Our study was the third to discuss the role of AMR in pancreas transplantation in 2010 (Chapter 3), and up until the publication of this thesis, only three other studies have followed[10;50;51]. One of the studies is a case report on a pancreas after kidney transplantation (PAK) recipient with AMR, TCR, and pancreatic panniculitis, with well-preserved graft function after a treatment with high-dose steroids[50]. The second study confirmed the differences between pancreas and renal allografts in terms of the rejection status after SPKT[10], following previous observations[12;13]. The third study addressed the formation of (donor-specific) anti-HLA antibodies in 167 pancreas transplantations. Twenty-six patients developed post-transplant DSA, which was found to be an independent risk factor for pancreas graft survival[51]. Histological parameters typical of AMR thus far include interacinar capillaritis, (inter-) acinar inflammation, thrombosis, and acinar cell and overall tissue injury (Chapter 3)[6]. In the study described in Chapter

4, 7 out of the 33 pancreas transplantectomy cases within 1 year after transplantation showed all 3 hallmarks of AMR. Furthermore, we found that AMR is associated with a poorer outcome of the pancreas graft (Chapter 3); however, larger cohorts need to be studied to confirm this observation. The possible clinical impact of these findings can only be determined by prospective studies. Once transplantation patients are treated based on the rejection status of their pancreas, we will have the opportunity to determine whether improvement of the graft and/or patient survival is feasible.

One reason for the small number of studies on rejection of the pancreas allograft is the shortage of pancreas allograft tissue samples, due to the reluctance to biopsy the pancreas. Most studies evaluate clinical parameters but fail to include any histological assessment, which is the gold standard in other transplant settings to assess rejection. Only a few large histological studies have been performed that discuss rejection and the role of AMR in thrombosis[52;53]. However, at the time those studies were conducted, C4d had not been recognized as a marker for antibody-dependent complement activation, and the role of *de novo* DSA was also not clear. At present, pancreas graft biopsy material is scarce. Pancreas allograft function is most often monitored by serum amylase, serum lipase, or (when bladder-drained) amylasuria[49]. Often the renal transplant biopsy is used as a surrogate for pancreas graft rejection status in SPKT. Our data, as outlined in Chapters 3 and 4, emphasizes the continuing importance of monitoring pancreas graft function by taking pancreas biopsies to distinguish immune and non-immune injury to the graft, as has been previously stated[54]. Especially considering that PAK becomes increasingly frequent due to an increase in living donor kidney transplantation, cases such as those reported by Carbajal *et al* may become even more abundant[55]. Carbajal *et al* reported a patient with PAK who developed AMR of the pancreas with a C4d-positive biopsy. This patient's kidney, which originated from a different donor than the pancreas, remained unaffected, but the pancreas graft was lost. Obviously, in the case of a PAK, a kidney biopsy will not be predictive of the clinical course of the pancreas graft because the organs originate from different donors. Our data emphasizes that, due to the level of discordance in the morphological findings between kidney and pancreas biopsies from the same donor, biopsies of both organs (when indicated) are likely justified, as has been previously shown[56]. However, prospective studies are necessary to ascertain whether intervention based on findings in either organ could improve allograft survival.

The reluctance to perform biopsies on the pancreas is based on a combination of factors. In SPKT, many physicians still believe that findings in the kidney biopsy are predictive of the processes taking place in the transplanted pancreas, in spite of the evidence against this assumption[10;12;13]. Therefore, there is the opinion that there is no legitimate reason to risk a biopsy of the pancreas. A biopsy of the pancreas is not without risk, but whether the assumed risk is equal to the actual risk remains to be determined. There is the fear of causing injury to the organ or inducing serious complications such as bleeding[11]. Several studies have addressed the mortality, morbidity, and risk of complications following percutaneous fine-needle aspiration in pancreatic neoplasms and pancreatitis. Mortality

and morbidity range from 0.006-0.08% and from 0.05-0.18%, respectively[57], and the incidence of major complications has been reported in 0.005-0.18% of fine-needle pancreas aspirations[58]. Although the risks are low, they are present, and death due to hemorrhagic pancreatitis induced by a percutaneous fine-needle aspiration is not only theoretical but has been described in a case-report[59].

In the pancreas transplant setting, the orientation of the pancreas is altered which hinders access, providing another reason for the lower number of biopsies taken after pancreas transplantation compared with pancreatitis and pancreatic neoplasms, and for the even fewer number of studies addressing the complications. A 1995 study reported the prolonged bleeding from the pancreas biopsy site (8%), clot retention (1%), and transient hyperamylasuria (20%) after the cytoscopically guided biopsy of the transplanted pancreas[11]. A 2000 study addressed pancreas transplant biopsies and found an adequate yield for the histopathological analyses in 83.3% of cases, regardless of the biopsy needle gauge and imaging used to guide the procedure. In a study that included 42 biopsies on 21 patients, 11% of the cases showed minor complications for all gauges and methods used. These minor complications comprised mild local bleeding (2%), air within the transplanted pancreas, and streaky density appearing adjacent to the biopsy site[60]. Boggi *et al* performed retroperitoneal pancreas transplantation with portal-enteric drainage and direct apposition of the pancreas body/tail to the lateral abdominal wall[61]. This method facilitates the percutaneous pancreas biopsy and results in 90% of biopsies being fit for diagnostic purposes (oral communication, Banff 2011 conference).

Studies from our lab and others indicate that rejection of the pancreas and kidney can take place discordantly in SPKT (Chapters 3 and 4)[10;12;13]. It is also generally known that the pancreas allograft has a poorer graft survival than the kidney allograft. Considering the risks associated with taking biopsies determined by the pancreatic neoplasm studies, the benefits may outweigh the risks in the pancreas transplant setting. The additional information acquired from histopathological analyses of pancreatic biopsy tissue may prove to be essential for the survival of the pancreas. To test this hypothesis, prospective SPKT biopsy studies should be initiated that compare current practice with the proposed practice of taking pancreatic biopsy tissue findings into account. This approach means that the immunosuppressive regimens adapted to just the findings in the kidney biopsy (as is often the current practice) should be compared to the immunosuppressive regimens adapted to the findings in both pancreas and kidney biopsies in order to assess survival advantage. In PAK and PTA, pancreatic biopsies will most likely be of even greater value, given that in this setting the pancreas is either the only allograft or has been derived from a different donor.

1.5 ß-cell replacement therapy (vascularized versus cell-based) and antibody-mediated rejection

Vascularized organ transplantation differs from cell-based transplantation in several aspects. In vascularized organ transplantation, only the donor endothelium is exposed to the recipient. In cell-based transplantation, various cell types and structures are directly

exposed to the recipient's blood and immune system. Islets of Langerhans transplantation differs from other cellular therapies, such as stem cell transplantation, as the islet itself is a miniature organ that needs to be revascularized in order to function properly. When the islet is extracted from the pancreas and transplanted, the previously embedded islet cell comes into direct contact with blood components. The normally embedded "outside" of the islet is known to express tissue factor (TF), a component driving thrombosis, after isolation[62]. Using a rodent islet isolation model, Saito *et al*reported that the induction of brain death, in combination with the warm ischemia time necessary for isolation of the islets, triggers the production of TF by islets[63]. When TF expressed on the cell surface comes into contact with blood, a process of immediate cell lysis known as the immediate blood-mediated inflammatory reaction (IBMIR) can be induced[62]. The co-infusion of heparin together with the islet transplant, which is directly infused into the bloodstream via the portal system of the liver, should dampen this effect[64]. When the islets have evaded IBMIR, they will not function properly until vascularized, which requires sufficient time[65]. Furthermore, the memory for autoantibodies directed against ß-cells that rendered the patient diabetic in the first place is still there and can provoke a new auto-immune reaction[66;67]. Consequently, many transplanted islets will be prematurely lost due to ischemic stress, apoptosis and necrosis.

2. ISLETS OF LANGERHANS TRANSPLANTATION

Currently, islet transplantation is not the cure that labile type 1 diabetes patients have been hoping for[68], and pancreas transplantation as a ß-cell replacement therapy surpasses islet transplantation in every aspect. There are several reasons why islet transplantation is currently not as successful as we would wish. This result is partly due to the late clinical onset of the treatment compared with other organ transplant settings. The first successful islet transplantation was reported in 1978, while the first reported successful pancreas transplantation was performed in 1966[69]. Vascularized pancreas transplantation is still undergoing improvements, as is islet transplantation. Only time will tell which of the two ß-cell replacement therapies will ultimately provide the most benefits to patients.

2.1 Obstacles in islet transplantation

Several theories on the causes of islet allograft failure after transplantation have been put forward, and some are discussed in Chapters 5 and 6, along with ideas regarding all pre-transplantation procedures and their effect on the yield and functionality of the isolated islets. The current protocols for islet isolation generate unsatisfactory low yields. At present, what is generally considered a highly successful isolation only yields 40% of all islets present in one pancreas, and often significantly fewer islets are isolated[70]. Several steps within the islet isolation procedure are currently under investigation. Studies are being conducted that are aimed at preserving larger numbers of functional islets in the excised pancreas, as many islets are lost during the transportation of the donor organ to

the Good Manufacturing Practice facility[71]. Furthermore, alternative isolation methods are being explored, such as magnetic retraction, instead of density gradient centrifugation.

Magnetic retraction involves the intra-arterial infusion of iron particles into the islet capillaries. In rats, this islet extraction method appears successful, and the iron particles infused in the islet and subsequently transplanted with the islet do not adversely affect the recipient[72]. Furthermore, the technique was faster than the original density gradient purification. This reduction in the ischemic period may result in improved functionality. In addition, the isolate was of higher purity, with less exocrine contamination[72]. This islet isolation protocol has also been applied in humans and pigs. The isolation of human pancreatic islets using iron particles was not as successful as the rodent islet isolation with iron particles, and neither the time nor the yield were improved when compared with density gradient purification[73]. Isolation of islets from pigs was more successful and had higher yields, although the yields were not as pure as was aimed for in the study[74]. Furthermore, the homogenous distribution of the iron particles throughout the pig's pancreas proved to be difficult, and the functional potency of the islets was not markedly improved[74]. Currently, once islets are isolated there is no means to predict the quality of the isolate in terms of *in vivo* functioning. The isolate is extensively tested to ensure quality standards as prescribed by the FDA and Good Manufacturing Practices, but this does not provide any indication of *in vivo* function. If a method to predict *in vivo* functioning existed, the amount of islets infused could be tailored for each patient, preventing the excessive use of islets while still providing adequate therapy. Numerous *in vitro* assays are currently being tested, and new assays are being developed to gain a better understanding of islet viability, potency, and function[75;76].

Although islets are infused into the liver in current practice (which has been shown to be successful in auto-islet transplantation[77]), the liver is presumably not the most optimal transplantation site. Alloislet transplantation faces many adverse complications, such as the risk of inducing an instant blood-mediated inflammatory reaction, induction of an hypoxic environment due to the lower oxygen tension and lack of vascularization in the liver, altered autonomic innervation, the energy status of the ß-cells, the balance of pro- and anti-apoptotic mediators, and the occurrence of ß-cell senescence[62;78-81]. Alternative sites have been explored (mostly in animal models), the most promising of which are the omentum and implants consisting of islets within a biomaterial structure (e.g., bio-artificial scaffolds[82] and microencapsulation[83]). Several factors need to be considered in the search for an optimal implantation site:

- Surgical and technical, as the operation should be minimally invasive and islets should be held within a restricted area for monitoring
- Cellular and metabolic, as oxygen should be readily available and metabolites and hormones have to be delivered to the vasculature
- Immunological, as most immunosuppressants are toxic for ß-cells and decreased direct blood contact potentially minimizes the chances for IBMIR[78].

Alternative sources of ß-cells are of interest because it is clear that the current lack of donor material hampers the success and employability of islet transplantation and will continue to do so. An advantage of islet transplantation over vascularized pancreas transplantation is the potential for an unlimited ß-cell supply through several possible strategies, such as stem cells[84], islet progenitor cells[85], xeno-islets[86], and the transition of other cell types such as duct cells[87], of which the latter seems promising. These research initiatives hold the future for islet transplantation, and the development of these alternative ß-cell sources is essential as there will always be a shortage of donor organs. To date, however, pancreas transplantation is favored over islets of Langerhans transplantation for almost all type 1 diabetes patients.

Another obstacle to implementing islet transplantation more widely (once it is successful) is the toxicity of the immunosuppressive regimen for ß-cells[88]. The kidney (native or transplanted) will also be damaged by most currently available immunosuppressive drugs, which in turn will negatively affect the islet allograft. To overcome this vicious circle of curing the patient through transplantation while inducing damage resulting in graft loss by immunosuppressive therapy, we explored methods to prolong allograft survival through cellular immune modulation. Cellular immune modulation may one day render immunosuppressive drugs obsolete. In this thesis, we show that donor-derived tolerogenic dexamethasone-treated dendritic cells (dexDC) were not capable of prolonging allograft survival and even showed an accelerated rejection most likely caused by antibody- and NK cell-mediated rejection (Chapter 7). This result will be expanded upon in the following section on cellular immune modulation.

The current success rates for islet transplantation are not as high as theory would predict. Eventually, almost all islet transplant recipients experience a reduction in functionality of their graft resulting in the reinstatement of exogenous insulin therapy, although at a lower dosage. A prerequisite to improving the success rate is to understand the underlying cause for islet allograft failure, both in the short and long term, thereby creating a better framework for intervention strategies. There are some clues from other transplant settings as to the causes of this graft failure[65]. For example, chronic allograft rejection may play a role[65], or the accumulating toxic effects of immunosuppressive drugs on ß-cells may induce islet transplantation failure at a later stage[88]. Recurrence of the underlying disease, type 1 diabetes, may also be potentially harmful. Furthermore, it has been reported that islet recipients with positive T-cell responses to auto-antigens were more likely to lose full graft function[67]. To assess failure, it is essential to monitor the graft. In other transplant settings that involve whole organs, a biopsy is regarded as the gold standard for diagnosis. Thus, performing a biopsy of the islet graft for monitoring would be preferred. Initial attempts to biopsy the liver in the hopes of attaining some liver engrafted islets revealed that almost all needle-biopsy cores do contain islets. However, the number of islets in the biopsy was so limited that it had no practical value for diagnosis[89]. It is, however, essential to visualize the graft to ascertain how to intervene and check whether the intervention was successful. New approaches using scaffolds consisting of islets within a biomaterial

will most likely reduce the side effects of the biopsy procedure and will ensure that all biopsy cores contain sufficient numbers of insulin-producing cells for diagnosis.

Other methods to visualize the transplanted islets have also been attempted. First attempts at the implantation of small amounts of syngeneic islet tissue in forearm muscle have provided a proof of principle that magnetic resonance imaging (MRI) would allow for insights into the processes occurring after transplantation[90]. Once the mechanisms of islet loss after transplantation are known, the possible means for intervention can be developed. Based on the preliminary findings in both human[90] and rodent models[91], the use of scaffolds and biomaterials to seed the insulin-producing cells seems promising. These approaches ensure that the islets will not be exposed to IBMIR, are easily accessible, and that engraftment and vascularization can be enhanced by supplement additions.

2.2 Immune modulation

Tolerance induction, or the creation of an accommodating environment for an allograft, is the "Holy Grail" of graft acceptance after allogeneic transplantation. Very few individuals are capable of acquiring this type of adaptive state by themselves[19;92]. The requirements for allograft tolerance are unclear, and much research has been conducted to understand the changes that have taken place in tolerant individuals. Once these adaptive changes have been elucidated, the requirements can be adopted and instated in new transplant recipients and would theoretically render them instantly tolerant. Several attempts have been made to create such an environment through various interventions at an early stage. Although blood transfusions were already known to potentially cause hyper-acute rejections, in 1978, Opelz and Terasaki[93] found that, in a large cohort of kidney transplantation recipients, those patients who received blood transfusions before transplantation had better graft survival than patients who did not receive transfusions. Later studies found that donor-specific transfusions were successful. However, with the discovery of cyclosporine A and the acquired knowledge that transfusions could lead to HLA sensitization, the use of transfusions in transplantation has been abandoned. Cellular immune modulation therapies have evolved from whole blood to specific components[94], such as dendritic cells or macrophages. More recently, recipient-derived cells have been modulated to become tolerogenic carriers of foreign (donor) antigens[95] to further fine-tune the system. Eventually, the goal is to make all immunosuppressive drugs obsolete, ensuring a non-toxic therapy for transplantation recipients without life-long compulsory and harmful drug administration, while achieving optimal graft function.

Cellular immune modulation has shown its potential in cancer therapy, although with limited success rates[96;97]. The problem in cancer therapy is that the targeted tissue is "self", and the tumor is effective at evading the immune system on which the cellular immune modulation therapy is based. In cancer, cellular therapy is only administered after all currently employed methods have failed. However, in the transplant setting, it is preferable to start cellular immune modulation therapy immediately after transplantation, given that this is the time when the immune response against foreign antigens is most active[98]. In addition,

the transplant setting has its own obstacles. Due to the necessary "non-self" targeting, it is unclear, for example, whether to use donor- or recipient-derived cells. In addition, it is unethical to test novel therapeutics without the use of concurrent regular maintenance immunosuppressive therapy. These drugs will most likely also alter the functionality of the cell-based therapy. The allogeneic transplanted tissue is not free from antigen-presenting cells (APCs) and lymphocytes, as these reside in the tissue, and additional effects of these co-transplanted cells are difficult to predict. In some animal models, tolerance induction in transplantation has been successful, but humans trials are just starting[98].

The hazards of cell-based therapeutic approaches are numerous. The tolerogenic status of the cell must remain intact, and *in vitro* studies cannot account for the full complexity of the *in vivo* situation. Furthermore, upon administration, the tolerogenic cells must be able to actually induce native immune cells of the host to become tolerogenic cells, resulting in full tolerance induction on location. As individuals differ in their make-up, the effects of the infused cell therapy will vary. One example of cellular immune modulation is the use of dendritic cells (DC) that have attained permanent immaturity[99]. In their immature state, DC have a tolerizing phenotype, which is capable of inducing regulatory T-cells. In theory, when a recipient is treated with permanently immature DC from the organ donor, a tolerant, accommodative environment may be created prior to transplantation. This would prevent the rejection of the allograft and render the use of immunosuppressive drugs obsolete. Dexamethasone-treated DC have been demonstrated to promote donor-specific T-cell hyporesponsiveness in two fully mismatched kidney transplant models[100]. As described in Chapter 7, we have tried to induce a tolerogenic state in a rodent islet transplantation model with the use of cellular immune modulation. The donor-derived tolerogenic dexamethasone-treated DC used were tested *in vitro* and shown to express reduced levels of CD86 and MHC class II molecules compared to non-tolerogenic DC after lipopolysaccharide stimulation. This result showed the preserved immature status of the dexDC even after a strong maturation signal was provided. Furthermore, the donor-derived dexDC have been shown to be capable of excreting IL-10, an anti-inflammatory cytokine, while lacking the ability to produce IL-12, a known pro-inflammatory cytokine. Therefore, the dexDC have a reduced capacity to stimulate allogeneic T-cells *in vitro* and have the capacity to induce T-cell hypo-responsiveness *in vivo*[100;101]. However, in our allogeneic rodent islet transplant setting, donor-derived dexDC actually accelerated rejection. As stated before, in theory these tolerogenic DC may induce a 'negative vaccination' resulting in increased alloimmunity[102], which we describe in Chapter 7. This does not imply that cellular immune modulation has no chance of becoming successful. Extensive research in all fields of organ transplantation and cancer therapy is still underway, and the knowledge gained will eventually lead to a workable treatment. In the end, DC may prove not to be the cell type of choice, as highlighted by the current heightened interest in macrophages[103].

3. CONCLUDING REMARKS

Acute rejection in organ and islet transplantation has many facets, and only a few have been touched upon in this thesis. In these concluding remarks, the main topics discussed are summarized.

In SPKT, immunosuppressive rejection therapy is currently administered based on the findings in the kidney allograft biopsy. However, we now know that the pancreas allograft may show a discrepant rejection profile compared with the kidney allograft, possibly requiring a different therapeutic regime. Furthermore, early graft survival rates of the pancreas lag behind the kidney allograft in these simultaneously transplanted patients. Prospective studies in which both kidney and pancreas biopsies are studied are necessary to discover whether pancreas-tailored therapy can improve the overall graft survival in this patient group. For PTA or PAK, the relevance of monitoring the pancreas graft through biopsies is even more essential, as in these cases, the kidney and pancreas are from different donors. We have established that not only TCR can take place in both organs but also that AMR is a valid type of rejection in the pancreas as well as in the kidney.

Islet transplantation will become a feasible ß-cell replacement therapy once it has improved sufficiently to overcome its current shortcomings. Theoretically, the islet transplantation process is less invasive and could be applied at a much earlier point than the current most successful ß-cell replacement therapy, which is whole pancreas transplantation. Regardless of the time these improvements may take, at present, there is no therapy available to prevent the onset of type 1 diabetes. Consequently, the demand for a cure, preferentially one relatively early in the progressive course of microvascular complications, is likely to increase further. The chronicity of the disease, the numerous secondary complications involved, and the increasing disease incidence will spur research efforts and bring ß-cell replacement therapy through islet transplantation closer to broad applicability.

Cellular immune modulation of the recipient is essential in ß-cell replacement therapy, as this transplant setting will remain ineffective if current immunosuppressive protocols are retained. In other transplant settings, improved tolerance of the recipient will also improve survival rates, and the efforts currently undertaken will not be in vain. Unfortunately, the same applies to cellular immune modulation as for islet transplantation; we are not there yet. However, the results are promising, and eventually research efforts will catch up with theory, making both cellular immune modulation and islet transplantation widely applicable and successful therapies.

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ADDENDA

NEDERLANDSE SAMENVATTING

Diabetes type 1 is een chronische auto-immuun ziekte, veroorzaakt door de zelfvernietiging van insuline producerende ß-cellen in de pancreas (alvleesklier). Ongeveer 15% van alle diabetes patiënten heeft diabetes type 1. Diabetes type 2 wordt veroorzaakt door een tekort in insuline productie om te compenseren voor de steeds toenemende insuline resistentie en obesiteit. In dit proefschrift gaat het alleen over diabetes type 1. Wanneer in deze samenvatting 'diabetes' is gebruikt, wordt de type 1 variant bedoeld.

Hoe deze ziekte ontstaat, is nog grotendeels onbekend en daarom zijn er ook nog geen therapeutische middelen om het ontstaan van diabetes type 1 tegen te gaan. Op dit moment is er maar één manier om patiënten met diabetes type 1 van hun ziekte te genezen: herstel van de ß-cel populatie door middel van transplantatie. Er zijn twee soorten transplantatie mogelijk om herstel van de ß-cel populatie te bewerkstelligen:

1. pancreastransplantatie, al dan niet gelijktijdig met een niertransplantatie, en
2. transplantatie van eilandjes van Langerhans.

In dit proefschrift worden pancreas-, eilandjes van Langerhans en niertransplantatie naast elkaar gelegd en wordt onderzocht hoe acute antilichaam gemedieerde afstoting hier een rol in speelt.

Pancreastransplantatie

Pancreastransplantatie wordt sinds 1966 uitgevoerd en is een erkende behandeling voor patiënten met diabetes. Bij pancreastransplantatie wordt de pancreas van een overleden donor uitgenomen en in de patiënt met diabetes overgezet. De patiënt kan op deze manier definitief van zijn diabetes genezen zijn. Hij zal geen dagelijkse insuline-injecties meer nodig hebben en niet meer blootstaan aan de ontwikkeling van secundaire complicaties van diabetes zoals retinopathie, neuropathie, cardiovasculaire schade en nefropathie. Deze laatstgenoemde secundaire complicatie heeft nierfalen tot gevolg en zorgt ervoor dat de patiënt afhankelijk wordt van dialyse. Wanneer deze patiënt in aanmerking komt voor een niertransplantatie kan er eventueel tegelijkertijd een pancreas getransplanteerd worden. Dit zorgt ervoor dat de onderliggende ziekte, diabetes, ook behandeld wordt en de nieuwe donornier niet zal blootstaan aan bovengenoemde diabetes-gerelateerde complicaties. Een transplantatie waarbij zowel de nier als de pancreas worden vervangen heeft een positief effect op de overleving van beide getransplanteerde organen en dat komt weer de gezondheid van de patiënt ten goede. Een pancreastransplantatie wordt daarom vaak in combinatie met een niertransplantatie uitgevoerd.

Om acute afstoting van het getransplanteerde orgaan tegen te gaan zal de patiënt levenslang immunosuppressieve medicijnen moeten nemen om het immuunsysteem te onderdrukken. Afstoting vindt plaats doordat het immuunsysteem de donororganen herkent als lichaamsvreemd en er zodoende alles aan doet om deze 'indringers' te verwijderen. Medicatie kan deze afstoting tegengaan, maar helaas zitten er ook nadelen aan deze medicijnen. Op lange termijn kunnen zij nierschade induceren. Daarnaast zijn de

insuline producerende ß-cellen erg gevoelig voor deze immunosuppressieve medicatie en kunnen zij erdoor beschadigd raken. Daarom is het belangrijk een goede balans te vinden tussen het tegengaan van afstoting enerzijds, en toxiciteit door geneesmiddelen anderzijds. Om dit te monitoren worden er protocollair, of op medische indicatie, biopten van het niertransplantaat genomen. Bij gecombineerde nier-pancreastransplantatie wordt vaak alleen naar een biopsie uit de nier gekeken en gaat men ervan uit dat dit voorspelt of er al dan niet afstoting plaatsvindt in het pancreastransplantaat. Aan de hand van de bevindingen in de biopten wordt geëvalueerd welke behandeling het meest geschikt is en wordt er gecontroleerd of de toegediende therapie ook het gewenste positieve effect heeft gehad.

Afstoting kan op twee manieren plaatsvinden: T-cel gemedieerde afstoting en antilichaam gemedieerde afstoting. T-cel gemedieerde afstoting werd het eerst ontdekt en de meeste therapieën die zijn ontwikkeld richten zich op T-cellen die schade induceren. Antilichaam gemedieerde afstoting is pas recentelijk ontdekt en na deze ontdekking duurde het enige tijd voordat het fenomeen ook klinisch werd geaccepteerd. De rol die antilichaam gemedieerde afstoting speelt, is ontdekt in getransplanteerde nieren en veel onderzoek hiernaar heeft zich dan ook op niertransplantatie gericht. Of antilichaam gemedieerde afstoting ook een rol speelt in de afstoting van andere getransplanteerde organen, wordt momenteel (onder andere in dit proefschrift) onderzocht.

Antilichaam gemedieerde afstoting wordt veroorzaakt door ontsteking van kleine vaatjes in het orgaan met infiltratie van neutrofielen – dit zijn witte bloedcellen- en door het achterblijven van moleculaire restanten van complementactivatie. Het complementsysteem is een onderdeel van het aangeboren afweersysteem. Het complementsysteem kan geactiveerd worden wanneer een antilichaam gericht tegen het donororgaan zich bindt aan het orgaan. Dit resulteert in een cascade van activatie componenten waarbij er een fragment van complementfactor 4, C4d, achter blijft als een soort "voetafdruk" van de activatie van het immuunsysteem. C4d kan vervolgens gevisualiseerd worden in het biopt door middel van immunohistologische technieken. De afstoting wordt systemisch gekenmerkt door antilichamen die zich specifiek tegen het donororgaan richten en niet aan het weefsel van de ontvanger kunnen binden. De combinatie van 3 componenten -histologische kenmerken, C4d-depositie in het weefsel en donorspecifieke antilichamen in het bloed- bepaalt of er sprake is van antilichaam gemedieerde afstoting in de getransplanteerde nier.

In hoofdstuk 2 worden de resultaten beschreven aangaande de prognostische waarde van 1 van de 3 componenten voor de diagnose antilichaam gemedieerde afstoting, C4d-depositie, voor niertransplantaat overleving. Deze studie is uitgevoerd in nierbiopten met histologische gedefinieerde acute rejectie episodes verkregen uit een populatie van transplantatie patiënten met een standaard immunologisch risico profiel. In deze studie beschrijven wij dat C4d niet op zichzelf kan voorspellen of er sprake zal zijn van steroid resistentie bij behandeling van de rejectie episode. Niertransplantatie patiënten met C4d-aankleuring in hun biopt hadden dezelfde patiënt- en niertransplantaatoverleving als de niertransplantatiepatiënten zonder C4d aankleuring in hun biopt. Daarnaast is er

geen verschil gebleken in de functionaliteit van de getransplanteerde nieren. In de studie beschreven in hoofdstuk 2 hebben we aangetoond dat C4d-depositie op zichzelf, in dit cohort biopten genomen binnen 6 maanden na niertransplantatie, niet gerelateerd is aan het klinische resultaat van transplantatie.

Over de rol van antilichaam gemedieerde afstoting bij pancreastransplantatie is nog weinig bekend. In het werk beschreven in hoofdstuk 3 is gekeken naar histopathologische laesies, C4d aankleuringspatronen en donorspecifieke antilichamen in relatie tot pancreastransplantaatoverleving. In deze studie beschrijven we dat de lange termijnsoverleving van het pancreastransplantaat slechter is bij die patiënten die in hun pancreastransplantaatbiopt een positieve interacinaire C4d-aankleuring lieten zien en bij wie in het serum donorspecifieke antilichamen gevonden werden. Deze resultaten zijn vergeleken met patiënten bij wie geen van bovenstaande kenmerken te zien waren in het pancreastransplantaatbiopt en serum. Verschillende specifieke histologische parameters bleken geassocieerd te zijn met de diagnose antilichaam gemedieerde afstoting, maar omdat er maar 28 biopten bestudeerd konden worden, is meer onderzoek nodig om de onderscheidende waarde van deze kenmerken te verifiëren. Wij concluderen dat zowel een diffuus positieve C4d-aankleuring als donorspecifieke antilichamen aanwezig moeten zijn om de diagnose antilichaam gemedieerde afstoting in het pancreastransplantaat te kunnen stellen.

In het onderzoek beschreven in hoofdstuk 4 is de studie uit hoofdstuk 3 uitgebreid door de histopathologische analyse van pancreastransplantectomiepreparaten. Biopsiemateriaal van de getransplanteerde pancreas is schaars en een biopt bestaat in de regel uit een zeer klein stukje weefsel. Materiaal van transplantectomieën is wel in overvloed voorhanden. In gecombineerde nier-pancreastransplantaties wordt vaak het niertransplantaat bij verdenking op afstoting gebiopteerd. Men neemt aan dat bevindingen in het nierbiopt ook voorspellen wat zich in het pancreastransplantaat afspeelt, zoals eerder vermeld. Het is echter al aangetoond, door zowel onze groep als anderen, dat deze aanname onjuist is. In gecombineerde nier-pancreastransplantaties is de pancreastransplantaatoverleving slechter dan de niertransplantaatoverleving. Het is bekend dat veel van de falende pancreata ten onder gaan aan technisch falen direct na transplantatie. Technisch falen gaat vaak gepaard met de ontwikkeling van trombose en interventiestrategieën zijn schaars. Aan het onstaan van trombose kunnen verschillende factoren ten grondslag liggen die mogelijk preventief of op indicatie behandeld kunnen worden.

Wanneer trombose binnen een jaar na transplantatie pancreastransplantaatverlies tot gevolg heeft, zou dat onder andere veroorzaakt kunnen zijn door antilichaam gemedieerde afstoting. Dit is onderzocht en beschreven in hoofdstuk 4. Wij hebben in deze studie alle pancreastransplantectomiepreparaten in het LUMC binnen een jaar na gecombineerde nier-pancreastransplantatie geïncludeerd (n=33). Deze zijn onderzocht en vergeleken met een controlegroep (n=66) waar het pancreastransplantaat minimaal 4 jaar functioneel was. Donorleeftijd en het geslacht van de ontvanger blijken geassocieerd te zijn met het falen van het pancreastransplantaat. In de pancreastransplantectomie preparaten is er zowel gekeken naar C4d-depositie in het weefsel, als naar donorspecifieke

antilichaamontwikkeling in het serum van de ontvanger. Van de 33 patiënten die binnen een jaar na transplantatie een pancreastransplantectomie ondergingen, waren er 7 patiënten met antilichaam gemedieerde afstoting. Van deze 7 patiënten met een gecombineerde nier-pancreastransplantatie, hebben er 6 hun nier binnen een jaar na transplantatie verloren, doch op een ander tijdstip dan het pancreastransplantaat verloren ging. Daarnaast was het kenmerkend dat het verlies van deze 7 antilichaam gemedieerde pancreastransplantaten op één na vóór 1994 plaatsvond. Sinds 1994 zijn er veel ontwikkelingen geweest in de samenstelling en het gebruik van immunosuppressieve medicatie. Dat zou mogelijk een verklaring kunnen zijn voor deze bevinding. Deze studie laat zien dat pancreastransplantaat verlies wel degelijk door antilichaam gemedieerde afstoting veroorzaakt kan worden, en dat dit histologisch vaak gepaard gaat met trombotische lesies.

Transplantatie van eilandjes van Langerhans

Transplantatie van eilandjes van Langerhans (eilandjes) is een recentere behandelmethode voor patiënten met diabetes, die nochtans alleen als onderzoekgerelateerde behandeling wordt toegepast. De pancreas bestaat voor 1 à 2% uit eilandjes van Langerhans. In die eilandjes bevinden zich de insuline producerende ß-cellen die defect zijn gegaan bij een patiënt met diabetes. Hoofdstuk 5 is een literatuurstudie die terugblikt op de opgedane kennis aangaande eilandjes van Langerhans transplantatie. Het gaat over de potentie die eilandjes van Langerhans transplantatie heeft, over de limitaties die uitgebreide succesvolle implementatie nog in de weg staan, en over mogelijke handreikingen voor behandelend artsen. Eilandjes van Langerhans transplantatie is een minder invasieve behandeling dan pancreastransplantatie en kan in theorie eerder na het ontstaan van diabetes toegepast worden dan een pancreastransplantatie. De 5-jaarsoverleving van het eilandjes van Langerhans transplantaat is momenteel 6.5%. Dit zal eerst verbeterd moeten worden voordat deze behandeling wijdverspreid toegepast kan worden.

Hoofdstuk 6 is een literatuurstudie die aangevuld is met eigen bevindingen over de histopathologische kenmerken van eilandjes van Langerhans transplantatieonderzoek bij ratten. De kenmerken waar we ons op gericht hebben in hoofdstuk 6 bestonden uit innervatie, vascularisatie door bloedvaten, en lymfatische vascularisatie van het eilandjestransplantaat onder het nierkapsel van de ontvangerrat. Eilandjes van Langerhans worden bij ratten onder het nierkapsel getransplanteerd. Dit verschilt van humane eilandjestransplantatie waarbij de eilandjes door infusie via de poortader vastlopen in de lever. De reden om de eilandjes van Langerhans bij de rat op een andere lokatie te transplanteren zijn divers. Eén reden is dat lokalisatie van de eilandjes onder het nierkapsel visualisatie vergemakkelijkt. Dit maakt de rat als proefdiermodel uitermate geschikt voor onderzoek naar eilandjes van Langerhans transplantatie. In het werk beschreven in hoofdstuk 6 wordt duidelijk dat, nadat de eilandjes van Langerhans zijn getransplanteerd, vele verschillende connecties tussen transplantaat en ontvanger hersteld moeten worden om de transplantatie tot een succes te maken. Het samenspel van alle componenten is essentieel en het is daarom onvoldoende slechts één aspect te bestuderen.

Zoals eerder beschreven zijn de insuline producerende ß-cellen in de eilandjes van Langerhans erg gevoelig voor immunosuppressieve therapie. Bij voorkeur zou er een alternatief voorhanden moeten zijn die huidige immunosuppressive medicatie overbodig maakt, bijvoorbeeld door de ontvanger van de eilandjes van Langerhans vóór transplantatie tolerant te maken voor de donor. De ontvanger zou dan de donoreilandjes van Langerhans niet meer als lichaamsvreemd zien, maar de eilandjes accepteren als "eigen" en op die manier kan afstoting worden verhinderd. In hoofdstuk 7 hebben wij getracht om door middel van celtherapie een tolerante omgeving te creëren bij de ontvanger van het transplantaat. Zodoende zou afstoting worden tegengegaan. Hiervoor is gebruik gemaakt van een proefdiermodel, waarbij één rattenstam fungeert als de eilandjes van Langerhans donor en een andere stam fungeert als diabetische ontvanger van het eilandjes transplantaat. Geïsoleerde eilandjes van verschillende donoren werden getransplanteerd onder het nierkapsel van één diabetische ontvanger. Wanneer de ontvanger normoglycemie heeft bereikt, kan de eilandjes-bevattende-nier verwijderd worden waarna de ontvangerrat weer diabeet zou moeten worden. Dit is een interne controle voor het induceren van diabetes en dient tevens om te bezien of de getransplanteerde eilandjes functioneel waren. De celtherapie om tolerantie te induceren bestaat uit dendritische cellen die uit de donorrat zijn geïsoleerd. Deze dendritische cellen zijn 7 dagen voor transplantatie bij de ontvanger ingespoten. De dendritische cellen zijn zo behandeld dat zij permanent immatuur zijn en een tolerante omgeving in de ontvangerrat zouden moeten kunnen creëren. De bevindingen beschreven in hoofdstuk 7 laten echter het tegenovergestelde zien. Behandeling van de ontvangerrat vóór transplantatie met permanent immature dendritische cellen van de donor, versnelt juist de afstoting van de eilandjes van Langerhans. Bij de ontvanger die behandeld is met de dendritische cellen zijn al vóór transplantatie donorspecifieke antilichamen te vinden. Deze kunnen, net zoals eerder beschreven, voor antilichaam gemedieerde afstoting zorgen. De snelheid waarmee de afstoting in dit model plaatsvindt wijst erop dat de afstoting veroorzaakt wordt door antilichaam-afhankelijke, cel-gemedieerde cytotoxiciteit. Hierbij zorgt de antilichaambinding niet voor complementactivatie maar voor directe cellysis door activatie van *Natural Killer* cellen. De celtherapie heeft niet het gewenste resultaat laten zien en meer onderzoek is nodig voordat het gebruik van celtherapie overwogen kan worden voor het humane eilandjes transplantatie veld. Eilandjes van Langerhans transplantie is dus nog niet zo succesvol als gewenst, maar stap voor stap wordt er meer kennis vergaard zodat dit uiteindelijk zal leiden tot wijdverbreide implementatie van deze behandeling.

CURRICULUM VITAE

Hanneke de Kort werd op 23 maart 1983 geboren in Tilburg. Na het behalen van haar VWO diploma in 2001 aan het Sint Odulphus Lyceum te Tilburg, begon zij haar opleiding biomedische wetenschappen aan de Universiteit van Amsterdam. Daar voltooide zij haar master-opleiding met twee afstudeeronderzoeken en een scriptie, aan de leerstoelgroep Swammerdam Institute of Life Sciences, Universiteit van Amsterdam, en de afdelingen Experimentele Vasculaire Geneeskunde en Medische Microbiologie, beide in het Academisch Medisch Centrum, Amsterdam in 2006. Tijdens haar studie was zij actief in de studievereniging CONGO en was zij een jaar lid van het dagelijks bestuur van die vereniging. In 2007 rondde zij haar tweede masteropleiding af in de medische antropologie en sociologie aan de Universiteit van Amsterdam, met een veldonderzoek in Wat Ma Klue, Thailand. Tijdens de laatste masteropleiding was zij werkzaam als analiste op de afdeling Experimentele Vasculaire Geneeskunde van het Academisch Medisch Centrum in Amsterdam. Ook nam zij aldaar zitting in de opleidingscommissie van de masteropleiding medische antropologie en sociologie. In 2007 begon zij aan haar promotie-onderzoek op de afdeling Pathologie van het Leids Universitair Medisch Centrum (LUMC), onder begeleiding van prof.dr. J.A. Bruijn, prof.dr. J.W. de Fijter, dr. E. de Heer en dr. I.M. Bajema. Van 2008 tot 2011 maakte zij deel uit van het bestuur van de promovendivereniging van het LUMC, de Vereniging (Arts) Onderzoekers. De resultaten van haar promotieonderzoek staan beschreven in dit proefschrift. Sinds 1 juni 2011 heeft Hanneke de Kort haar loopbaan voortgezet met een long-term ERA/EDTA postdoc fellowship bij de groep van prof.dr. H.T. Cook in het Hammersmith Hospital, Imperial College, London.

Hanneke de Kort was born in Tilburg, the Netherlands on March 23, 1983. After graduating secondary school in 2001 at Sint Odulphus Lyceum in Tilburg, she went on to study biomedical sciences at the University of Amsterdam, the Netherlands. She completed her master studies in 2006 with two graduation research projects and a thesis at the Swammerdam Institute of Life Sciences, University of Amsterdam, the department of Vascular Medicine, Academic Medical Center, Amsterdam, and at the department of Medical Microbiology, Academic Medical Center, Amsterdam, respectively. During this period, Hanneke was an active member of the association for bio(-medical) students and was part of the board of that association in 2003. In 2007 she obtained a master degree in medical anthropology and sociology at the University of Amsterdam, with a graduation project in Wat Ma Klue, Thailand. During her last master studies, Hanneke worked as a technician at the department of Vascular Medicine, Academic Medical Center, Amsterdam. In addition, she was part of the educational committee of the master studies medical anthropology and sociology. At the end of 2007 she started her PhD research under supervision of prof. J.A. Bruijn, prof. J.W. de Fijter, dr. E. de Heer, and dr. I.M. Bajema at the department of Pathology at the Leiden University Medical Center (LUMC), Leiden, the Netherlands. From 2008 to 2011 she was a board member of the LUMC PhD fellows' society. The results of her research are described in this thesis. Since June 2011 Hanneke has joined the group of prof. H.T. Cook in Hammersmith Hospital, Imperial College, London, with a two-year long-term ERA/EDTA-funded fellowship.

ABBREVIATION LIST

ADCC	antibody-dependent cellular cytotoxicity
AMR	antibody-mediated rejection
APC	antigen presenting cells
CDC	complement dependent cytotoxicity
DAF	decay accelerating factor
DC	dendritic cells
dexDC	dexamethasone-pretreated dendritic cells
DSA	donor-specific antibodies
ENDAT	endothelial activation transcripts
ESRF	end stage renal failure
FDA	food and drugs administration
GSTT1	Glutathione S-transferase theta-1
HLA	human leukocyte antigen
HUVEC	human umbilical vein endothelial cell
IAC	interacinar capillaries
IAK	islet after kidney transplantation
IBMIR	instant blood-mediated inflammatory reaction
IPTR	International Pancreas Transplant Registry
ITA	islet transplantation alone
LUMC	Leiden University Medical Center
MAC	membrane attack complex
MCP	membrane cofactor protein
MHC	major histocompatibility complex
MICA	human major histocompatibility complex class I chain-related A protein
MICB	human major histocompatibility complex class I chain-related B protein
MRI	magnetic resonance imaging
NK	natural killer
PAK	pancreas after kidney transplantation
PTA	pancreas transplantation alone
PTC	peritubular capillaries
SPKT	simultaneous pancreas kidney transplantation
TCR	T-cell mediated cellular rejection
TF	tissue factor

LIST OF PUBLICATIONS

1. **de Kort H**, Willicombe M, Brookes PA, Dominy K, Santos-Nunez E, Galliford J, Chan Kk, Taube D, McLean AG, Cook HT, Roufosse CA. Microcirculation inflammation associates with outcome in renal transplant patients with de novo donor-specific antibodies. *Am J Transplant*; accepted for publication.

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DANKWOORD

In de Dikke Van Dale staat het volgende:

pro·mo·ve·ren *(werkwoord; promoveerde, heeft, is gepromoveerd)*
1 de academische graad van doctor verwerven
2 naar een hogere rang of klasse overgaan

Eigenlijk had bij deze beschrijving ook nog moeten staan dat je promoveren niet alleen doet. Zonder de hulp van velen was dit boekje namelijk nooit tot stand gekomen en was de weg naar het promoveren ook een stuk minder gezellig geweest. Het promotieproces is er één met diepe dalen en hoge pieken en het is fijn dat er te allen tijde mensen waren om dit samen met mij te beleven. Daarom een woord van dank aan mijn helden (de volgorde doet overigens niets af aan de geleverde bijdrage).

Ingeborg, nog nooit heb ik iemand zo hard en zo veel zien werken. Het was een voorrecht dat je ook voor mij tijd vrij kon maken. De Banff in Banff was zeker een hoogtepunt en ik hoop dat wij nog veel artikelen samen mogen publiceren. Emile, met veel enthousiasme en uiteindelijk succes, hebben we samen het eilandjes transplantatieproject weer opgepakt. Ook wanneer er twijfels of tegenslagen waren heb je me gesteund en daar ben ik je erg dankbaar voor. JA, altijd gefocust op het boekje, deadlines en de grote lijnen. Je nauwkeurigheid, eindeloze inzet en de manier waarop je heldere vraagstellingen weet te formuleren, zijn een bron van inspiratie. Hans, bedankt dat ook jij je kostbare tijd hebt weten vrij te maken om mij te begeleiden. Jouw kennis en input waren onmisbaar. Daarnaast waardeer ik je algemene betrokkenheid bij mijn carrière, die verder gaat dan alleen dit proefschrift.

Iedereen op de PA heeft zijn of haar steentje bijgedragen en het zal niet lukken om jullie allemaal persoonlijk te vermelden maar je weet zelf wel dat ik jou bedoel! Vooral de oude garde heeft een plekje in mijn hart veroverd. Mijn directe collega's, de NePa's, dank aan jullie allen voor de gezelligheid, de hulp, het sparren, de sushi-momenten en alle input bij de besprekingen. Jullie waren met velen: Aletta, Ana, Annelies, Antien, Arda, Astrid, Bart, Danielle, Daphne, Dorrith, Gerrit, Hans, Idske, Jamie, Klaas, Lisette, Malu, Marian, Marije, Marlies, Mathilde, Mirjam, Prof. van Es, Ramzi, Rob, Suzanne, Thijs en natuurlijk niet te vergeten Diego! Lisa, heel veel succes met je vervolg studie en carriere.

Annemieke zonder jou was geen enkel experiment gelukt; van falende insulinekleuringen tot rattenreanimaties, jij wist alles en iedereen te redden. Dank voor alle gezelligheid en wijsheid en ik mis je.

Dank aan alle co-auteurs voor jullie inzet, de islet meeting groep voor de goede en gezellige besprekingen, de patiënten voor het meewerken aan onderzoek en natuurlijk dank aan mijn VAO-buddies voor de nodige afleiding en het grotere geheel. Ook dank aan alle mensen op beide stageplekken. Ik kan niet iedereen opnoemen, want dan vergeet ik er

zeker een paar, maar jullie hebben me laten zien wat onderzoek doen zo ontzettend leuk maakt en hoe je daarnaast ook nog borrelen kan. Jurgen en Stefan, dank dat jullie tijd in mij hebben willen investeren naast jullie drukke eigen onderzoek.

Some acknowledgements need to be in English for my new colleagues at the CCIR. Thank you all for being there and lending your ear in times of desperation. Even though the Dutch PhD process is a different one, the stress and struggles it comes with, are interchangeable and it has helped to know that in the end all will be fine.

De LMA, wat fijn dat we al die tijd onze etentjes erin hebben weten te houden en dat er naast al die doktoren ook nog promoverende biologen zijn overgebleven die met precies dezelfde frustraties en obstakels te kampen hebben (gehad). Ik kom snel weer jullie kant op om mee te theeën.

De Gèsselse meiden Yvet, Lotte, Marlon en Linda, dank dat jullie mijn vriendinnen willen zijn. Laten we ons 30ste levensjaar met een knaller inluiden, eindelijk met Honey starten, de halve marathon in NY lopen, alle bedachte reizen uitvoeren, en samen oud genoeg worden om het bejaardenhuis onveilig te maken. Jullie maken het leven leuk!

Lieve oma altijd geïnteresseerd hoe het met de muizen ging en of het boekje nu eindelijk klaar is. Nou hier is het boekje dan, nu alleen nog lezen.

Pap, mam, Frits en Yvette het is af hoor, niet dat jullie daar nu over in hebben gezeten. Nu op avontuur in London waar weer een buitenhuisje wacht dat jullie altijd zal verwelkomen en binnenkort kunnen jullie eindelijk weer naar jullie favoriete stek in Amsterdam!