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Mechanisms of Neonatal Porcine Islet Xenograft Rejection

by

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ABSTRACT

Islet transplantation has the potential to be an effective treatment for patients with type 1 diabetes. However, a shortage of human donor islets and the need for continuous immunosuppressive therapy currently limit this therapy to patients with brittle type 1 diabetes. Neonatal pigs may provide an unlimited source of islets for transplantation; however, the barrier of islet xenograft rejection must still be overcome. Understanding the mechanism of neonatal porcine islet (NPI) rejection will help to develop targeted therapies to prevent rejection. This thesis studied the early immune cells and molecules involved in NPI xenograft rejection, compared the role of NK cells in two models of islet xenotransplantation and investigated the role of T cell co-stimulatory and adhesion pathways in NPI xenograft rejection. Targeting these aspects of the immune response to NPI xenografts with short-term therapies may play a role in improving NPI xenograft acceptance and induce long-term xenograft survival.

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TABLE OF CONTENTS

CHAPTER ONE

PROGRESS IN ISLET XENOTRANSPLANTATION FOR THE TREATMENT OF TYPE 1 DIABETES MELLITUS

CHAPTER TWO

IMMUNE CELLS AND MOLECULES INVOLVED IN THE EARLY EVENTS OF NEONATAL PORCINE ISLET XENOGRAFT REJECTION

CHAPTER THREE

DIFFERENTIAL ROLE OF NATURAL KILLER CELLS IN RAT VERSUS NEONATAL PORCINE ISLET XENOGRAFT REJECTION IN IMMUNE-COMPETENT MICE

CHAPTER FOUR

COMBINATION OF CTLA4-Ig AND MONOCLONAL ANTIBODY THERAPY PREVENTS THE REJECTION OF NEONATAL PORCINE ISLET XENOGRAFTS IN IMMUNE-COMPETENT MICE

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

αGal	galactosyl alpha (1-3) galactose
Ab	antibody
ABC/HP	avidin-biotin complex/horseradish peroxidase
ADCC	antibody-dependent cell mediated cytotoxicity
APC	antigen presenting cell
B6	C57BL/6J
B7RP-1	B7-related protein-1
BG	blood glucose
cDNA	complementary deoxyribonucleic acid
CCR	CC chemokine receptors
CD	cluster of differentiation
CTLA-4	cytotoxic T-lymphocyte antigen-4
DAB	3,3-diaminobenzidinetetrahydrochloride
DM	diabetes mellitus
DSG	15-deoxyspergualin
ELISA	enzyme linked immunosorbent assay
ESC	embryonic stem cell
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FICC	fetal islet-like cell cluster
FITC	fluorescein isothiocyanate
GAPDH	glutaraldehyde 3-phosphate dehydrogenase
GLUT-4	glucose transporter-4
GMP	good manufacturing practice
GnT-III	N-acetylglucosaminyltransferase-III
HBSS	Hank's buffered salt solution
HLA	human leukocyte antigen
IBMIR	instant blood mediated inflammatory reaction
ICAM	intercellular adhesion molecule
ICOS	inducible T cell costimulator
IE	islet equivalent

IgG	immunoglobulin G
IFN-γ	interferon gamma
IL	interleukin
i.p.	intraperitoneal
i.u.	international units
kD	kilodalton
LFA-1	lymphocyte function-associated antigen-1
mAb	monoclonal antibody
MAC	membrane attack complex
MCP-1	monocyte chemotactic protein-1
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein-1 alpha
MIP-1β	macrophage inflammatory protein-1 beta
mRNA	messenger ribonucleic acid
NK	natural killer
NKT	natural killer T-cells
NOD	non-obese diabetic
NPI	neonatal porcine islets
OCT	optimal cutting temperature
PBS	phosphate buffered solution
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
Pdx-1	pancreatic and duodenal homeobox-1
PE	polyethylene
PERV	porcine endogenous retrovirus
PP cell	polypeptide cell
rag	recombination activating gene
RANTES	regulated upon activation normal T cell expressed and secreted
RNA	ribonucleic acid
RT	reverse transcriptase
SCID	severe combined immunodeficiency
TCR	T-cell receptor

TGFβ	transforming growth factor beta
Th	T helper
U	units

CHAPTER ONE

PROGRESS IN ISLET XENOTRANSPLANTATION FOR THE TREATMENT OF TYPE 1 DIABETES MELLITUS

1.1 DIABETES MELLITUS

Diabetes mellitus (DM) is a group of diseases which share the common clinical presentation of hyperglycemia (1). Two classes of DM are differentiated by distinct pathophysiologic processes which lead to a common hyperglycemic presentation. Type 1 DM, typically of juvenile onset, is characterized by the absence of insulin production (2). Two subgroups of type 1 DM exist: The more prevalent Type1a DM, is an autoimmune disease resulting in pancreatic β cell destruction. Type 1b DM, in contrast, lacks the presence of autoimmune markers yet presents with destruction of insulin secreting β cells by an unknown mechanism (2). Type 2 DM, on the other hand, can be caused by one or a combination of the following pathophysiologic processes: insulin resistance, impaired insulin secretion and/or increased glucose production leading to hyperglycemia (1). Type 2 DM most commonly presents during adulthood, however, the incidence of juvenile onset type 2 DM is increasing. Between 8-45% of new juvenile diabetics are being diagnosed as type 2 diabetics, depending on their location worldwide (3).

Diabetes mellitus induced chronic hyperglycemia can result in severe and debilitating diseases to a number of organ systems (1). Secondary complications of chronic hyperglycemia can be divided into non-vascular and vascular complications (1). Nonvascular complications include gastroparesis, infections and skin changes (1). Vascular complications, which represent the majority of the morbidity and mortality associated with the DM, can be further sub-classified: Microvascular complications, which manifest as retinopathy, neuropathy or nephropathy, and macrovascular complications, which manifest as coronary artery disease, peripheral arterial disease or cerebrovascular disease (1).

1.2 THE IMPACT OF DIABETES MELLITUS

Diabetes mellitus has a negative impact on both individual patients and society as a whole. Diet modifications, monitoring of blood glucose levels, administration of medications and the burden of chronic disease result in decreased quality of life among diabetic patients (4) while DM induced complications limit patients' ability to earn income (5). On a societal level, DM has significant implications on health care spending. Over 2 million Canadians live with diabetes, which accounts for an estimated $1.6 billion in health care costs; 25% is attributed to direct, medical expenditure, whereas 75% attributed to loss of production due to morbidity and mortality associated with the disease (6). Effective treatments to prevent DM and its complications are therefore critical to control the rising individual and societal costs associated with this disease.

1.3 PATHOPHYSIOLOGY OF TYPE 1 DIABETES MELLITUS

1.31 INSULIN

Key to the pathophysiology of DM is insulin, a 5800 kD globular protein produced and secreted by the β cells of the pancreatic islets (7). The structure of insulin has been highly conserved through vertebrate evolution such that human and porcine insulin differ only by a single amino acid at the carboxy-terminal of the B chain (Figure 1-1) (8, 9). As a result, the 3-dimensional structure of insulin is conserved across these species (7).

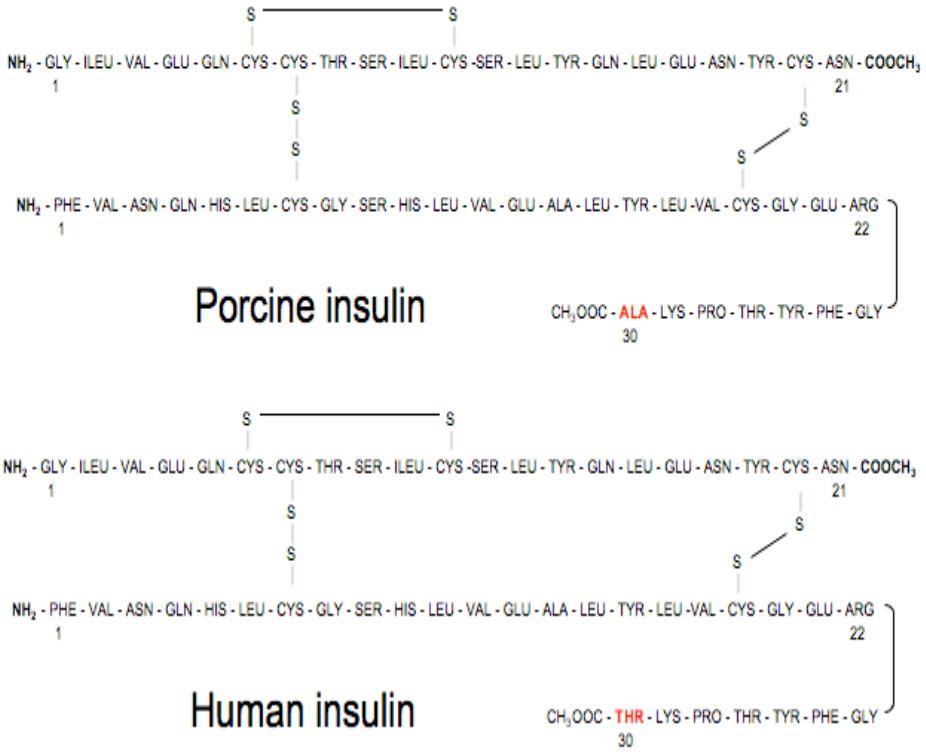

Figure 1-1: Comparison of porcine and human insulin amino acid sequences. Both porcine and human insulin consist of 2 polypeptide chains – a 21 amino acid alpha chain and a 30 amino acid beta chain. Porcine insulin (upper structure) differs by a single amino acid (ALA vs. THR) at position 30 of the beta chain. (Adapted from Ruttenberg, Science 1972, 177:623-626)

Translation of insulin mRNA results in the production of a precursor molecule, preproinsulin, which is a single chain 110 amino acid molecule (7). Once transported to the rough endoplasmic reticulum, preproinsulin undergoes cleavage and is folded to form a stable precursor, proinsulin (7). Proinsulin is packaged into secretory granules where it undergoes further modification to form insulin and a 31 amino acid residue, C-peptide (7). Because proinsulin is cleaved within secretory granules, insulin and C-peptide are present in a 1:1 ratio – a relationship that is clinically significant in the quantification of insulin secretion. Insulin has a rapid plasma half-life and undergoes concentration dependent endocytosis and degradation in peripheral tissue making direct quantification

of insulin difficult (7). In contrast, C-peptide has a longer plasma half-life and is cleared by the kidneys in a concentration independent manner. As a result, quantification of insulin secretion can be accurately measured indirectly by plasma C-peptide levels (7).

Insulin secreted by pancreatic β cells enters the portal venous system where approximately 50% is immediately metabolized by the liver (1). Insulin which has reached the systemic circulation has multiple metabolic and mitogenic effects on insulin sensitive cells. Insulin binds to insulin receptors found on the surface of insulin sensitive cells resulting in tyrosine-kinase activation (1). Depending on the target tissue, the effect of insulin signalling has different outcomes. In muscle, fat and liver cells, insulin signalling results in glucose transporter (GLUT-4) translocation to the cell surface. As a consequence, glucose is transported into these cells for metabolism and/or storage thereby regulating blood glucose concentrations (10). Insulin also plays an anabolic role in the body helping to promote the storage of substrates in muscle fat and liver cells by promoting glycogen synthesis, protein synthesis, lipogenesis, and stimulating cell growth and differentiation (1, 10).

1.3.2 THE ISLET CELL

Working as a medical student in Virchow's laboratory in 1869, Paul Langerhans was the first to describe "small cells of almost perfect homogeneous content and of a polygonal form" scattered amongst the acinar glandular cells of the pancreas (11). Nearly 30 years later, Laguesse postulated that these cells produced an internal secretion, and coined them as "ilots de Langerhans" (11). In humans, islet cells supply all of the pancreatic endocrine secretions, despite being only 1-2% of the total pancreatic mass

(12). The islet cell is further comprised of four cell types: insulin secreting beta cells (β cells), glucagon secreting alpha cells (α cells), somtatostatin secreting delta cells (δ cells) and pancreatic polypeptide secreting pancreatic polypeptide cells (PP cells). Rodent islet cells are comprised of predominantly β cells which are arranged in a clustered core surrounded by a mantle of the other three cell types (13, 14) and surrounded by a thin capsule composed of a single layer of fibroblasts and collagen fibres (12). Small arterioles direct blood flow first through β cells, then subsequently to α and δ cells, before entering the portal venous system which is drained into the liver (13). The cytoarchitecture of human islet cells, long believed to be similar to that of rodents, has now been elucidated with the use of confocal microscopy (15). Cabrera and colleagues have demonstrated that human islet cells contain proportionally fewer β cells and more α cells than mouse islet cells. Additionally, in human islet cells, endocrine cell types are arranged along blood vessels with no particular order or organization and as a result cellular arrangement within the human islet cell does not impact its function (15).

1.3.3 AUTOIMMUNE DESTRUCTION OF ISLET CELLS

The development of type 1 DM is a multifactorial process involving both genetic factors and environmental factors which leads to activation of autoimmunity. Several loci within the HLA region of chromosome 6 have been found in patients predisposed to type 1 DM. Genotyping of the HLA loci has demonstrated that HLA DR3 and DR4 haplotypes are most frequently encountered in type 1 DM patients compared to the normal population (1). Monozygotic twin studies have demonstrated that type 1a DM

concordance ranges between 30 and 70% (1, 16); thus suggesting that environmental factors also play an important role in type 1 DM pathogenesis.

Despite a genetic predisposition to type 1 DM, it is postulated that an immunologic trigger initiates the autoimmune process which leads to the destruction of islet β cells (1). Numerous immunologic triggers have been proposed, however, direct causation has yet to be established for type 1 DM (16). Enteroviral infections, breastfeeding, the early presence or absence of certain foods, birth weight, childhood over-nutrition, maternal islet autoimmunity, and negative stressors have all been correlated with the prevalence of type 1 DM through observational studies (17, 18). Proving direct causation has been difficult, however, given confounding exposures and genetic susceptibility in this study population (17).

In the non-obese diabetic (NOD) mouse model of autoimmune diabetes there are multiple auto-antigens which play a role in the pathogenesis of DM (18). While β cell specific auto-antigens, such as insulin and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), are targeted by auto-reactive immune cells, a host of non β cell specific auto-antigens are also implicated in the pathogenesis of type 1 DM (18). In both humans and NOD mice, autoimmune destruction of β cells is characterized initially by peri-insulitis, a non-pathogenic infiltration surrounding the islets of $CD4^+$ and $CD8^+$ T cells, B lymphocytes, macrophages, dendritic cells and other bone marrow-derived cells (19). Subsequently, insulitis occurs where $CD4^+$ and $CD8^+$ T cells invade the islets and induce apoptotic destruction of β cells (20). Adoptive transfer experiments in NOD-SCID mice have shed some light on the cellular pathogenesis of autoimmune diabetes. Alone, adoptively transferred purified $CD4^+$ T cells from diabetic NOD mice

have been demonstrated to induce diabetes in immune deficient NOD-SCID mice. However, when purified $CD4^+$ T cells from prediabetic NOD mice are adoptively transferred to immune deficient NOD-SCID mice, insulitis does not ensue (21). While $CD4^+$ T cells may themselves induce β cell death in the end-stages of autoimmune diabetes, possibly through the secretion of cytokines (16), it appears that early stages of type 1 DM pathogenesis are dependent on the presence of $CD8^+$ T cells (22, 23). Through adoptive transfer experiments, Serreze and colleagues were able to demonstrate that splenocytes from prediabetic NOD mice were capable of inducing autoimmune diabetes in MHC class 1-positive, but not MHC class 1-negative NOD-SCID mice – demonstrating the central role of $CD8^+$ T cells in autoimmune diabetes pathogenesis (23). Furthermore, a role for NKT cells has also been identified in the pathogenesis of type 1 DM. The NOD mouse model of autoimmune DM is deficient in the NKT cell population, however, the augmentation of this cell population through adoptive transfer has been demonstrated to suppress the development of diabetes (24). It is likely that NKT cells play a role in regulating T cell responses given that activated NKT cells are capable of suppressing diabetogenic $CD8^+$ T cells in NOD mice and are also capable of suppressing both Th1 and Th2 cytokine production in this animal model of type 1 DM (24).

1.4 TREATMENT OF TYPE 1 DIABETES

1.4.1 INSULIN REPLACEMENT THERAPY

Prior to the discovery of insulin, type 1 DM was a disease with very high mortality. Treatments were limited and primarily aimed at managing diabetic

ketoacidosis through starvation and carbohydrate restricted diets (25). In 1922, Fredrick Banting and Charles Best discovered insulin while working in the laboratory of John McLeod at the University of Toronto by demonstrating the ability of a crude pancreatic extract to reverse diabetes in pancreatectomized dogs (25). Instrumental in the discovery of insulin was the work of J.B. Collip, a biochemist from the University of Alberta. Collip's expertise in biochemistry allowed him to isolate the active molecule in Banting and Best's pancreatic extract - insulin. Using the purified insulin generated by Collip, Banting and Best were able to demonstrate the effectiveness of insulin in treating human patients with diabetes (25). In 1923, under controversial circumstances, Banting and McLeod were awarded the Nobel prize in medicine for their discovery of insulin.

Since Banting's discovery in 1922, insulin has become vital in treating both patients with type 1 and type 2 DM. Due to the similar structure across species, bovine and porcine insulin have been extracted and used in the treatment of human diabetics (7, 25). Despite their similarities, occasionally complications were encountered from animal insulin, such as skin rashes (26). In 1978, insulin became the first recombinant human protein to be manufactured (26) and is now used exclusively in the treatment of human diabetics.

A combination of dietary modification and subcutaneous insulin delivery remains the standard therapy for patients with type 1 DM. Advances in insulin formulation continue to strive towards the optimal combination of basal and bolus administration to regulate blood glucose levels. Short-acting insulin formulations are designed to be rapidly absorbed and coincide with post-prandial spikes in blood glucose (BG) levels (27), while long-acting insulin formulations are aimed at controlling basal BG levels.

New formulations, including inhaled insulin are designed to be rapidly absorbed and timed with meals (27). The safety and efficacy of inhaled insulin has been established (28) but clinical trials examining the ability to tightly control BG levels using this delivery system are ongoing. An insulin pump provides a stable basal delivery of insulin; continuous subcutaneous insulin infusion is effective in decreasing the frequency of hypoglycaemic events (29), however, these devices are subject to potential malfunction and are more resource intensive than standard multiple injection insulin therapy (29).

Since the advent of insulin, death from diabetic ketoacidosis is a rare event. While stringent glycemic control with insulin therapy can reduce the incidence of secondary microvascular complications (30), this requires life-long adherence and increases the potential for hypoglycaemic episodes. Rigorous monitoring and insulin therapy neither guarantees normal glycosylated hemoglobin levels, nor prevents microvascular complications (30). Due to the inability to control the development of secondary microvascular complications with exogenous insulin therapy, more physiologic means of delivering insulin through β cell replacement via pancreatic or islet cell transplantation have been developed (13, 31).

1.4.2 PANCREATIC TRANSPLANTATION

Following Brooks and Gifford's initial description in a large animal model in 1959 (32), advances in whole pancreas transplantation lead to transient reversal of hyperglycemia in a canine model (33). Given the favourable results seen in animal models, the first series of clinical whole pancreas transplant were performed at the University of Minnesota in 1966 (33). This initial series of whole pancreas

transplantation were complicated by leakage of pancreatic exocrine secretions and transplant rejection (31, 33). In the 1980s, immunosuppression with cyclosporine and bladder drainage of the exocrine pancreas were introduced, with subsequent improvements in pancreatic transplant outcomes (31). Despite the morbidity associated with this major surgery, successful pancreatic engraftment results in euglycemia and normalization of glycosylated hemoglobin levels (34). In the current era, morbidity associated with pancreatic transplantation ranges from 7.0% to 11.5% (35). All-cause mortality in transplant wait-list patients receiving conventional treatment, is equivalent to that of patients receiving simultaneous pancreatic transplantation and renal transplantation for end-stage diabetic nephropathy, but significantly worse for patients receiving pancreatic transplantation alone or after kidney transplantation (35). Due to its relatively high morbidity, pancreatic transplantation is primarily offered to uremic patients who are undergoing simultaneous renal transplantation (31).

1.4.3 ISLET TRANSPLANTATION

The very first attempts at islet transplantation occurred prior to the discovery of insulin in 1922. In 1892, Von Mering and Minkowski demonstrated that the pancreas was responsible for regulation of blood glucose as pancreatectomized dogs became hyperglycemic (36). Minkowski subsequently attempted to reverse diabetes in these diabetic dogs by autotransplanting fragments of the pancreas subcutaneously (37). In 1893, Williams attempted to treat a young diabetic boy with subcutaneous implants of sheep pancreas fragments; while there was a temporary improvement in his glycosuria,

the sheep pancreas xenograft was ultimately rejected and the boy died shortly thereafter (38).

Subsequent advances in rodent models established the foundation for techniques used in current day islet cell transplantation. In 1964, Hellerström demonstrated the ability to isolate islets for transplantation using a microdissection technique (39). While valuable as a proof of concept, microdissection of islets was both traumatic and yielded too few islets for transplantation. The combined use of collagenase with mechanical force to disrupt the pancreas and liberate a greater proportion of islets for transplantation was first described by Moskalewski in 1965 (40) using chopped guinea pig pancreas. While this technique of enzymatic digestion did result in complete separation of islets from the pancreatic acini, many of the islets were destroyed in the process. In 1967, Lacy and Kostianovsky (41) further refined this technique by distending the pancreatic duct prior to morcellation and collagenase digestion of rat pancreases. Using this technique, Lacy and Kostianovsky were able to isolate 300 islets from a single rat pancreas, demonstrating for the first time that an adequate number of viable islets could be isolated for transplantation. In 1973, Scharp and colleagues refined the isolation of islets by substituting the use of a sucrose density gradient with a dialyzed Ficoll density gradient to separate unwanted acinar debris from rat islets (42). Ficoll, which was felt to provide a better osmotic environment for the islets compared to sucrose, varied in its ability to isolate viable islets from lot to lot. However, by dialyzing ficoll to remove contaminants, Scharp and colleagues were able to demonstrate the ability to produce a high yield of viable rat islets for transplantation (42).

The first attempt at transplanting isolated rodent islets was performed by Younoszai and colleagues (43) and resulted in a temporary amelioration of hyperglycemia in a diabetic rat model. The first sustained reversal of chemically induced diabetes in a rodent model, however, was demonstrated by Ballinger and Lacy in 1972 (44). In this experiment, 400-600 isolated rat islets were transplanted either into the peritoneal cavity or in the thigh muscle of rats. Further substantiating their results was the fact that when the islet isograft was removed from the thigh muscle site, the recipient rats returned to a hyperglycemic state (44). The introduction of intraductal infusion of collagenase (45, 46), which permits collagenase to more effectively digest surrounding connective tissue, permitted a greater yield of isolated islets. These early small animal experiments demonstrated that the concept of β cell replacement by transplantation of isolated endocrine islets was indeed possible and opened the door for experiments to come.

Several sites of transplantation have been investigated through the course of islet cell transplant development. Transplantation into intramuscular and intraperitoneal sites by Ballinger and Lacy (44) were successful in reversing diabetes, however, consistent results were difficult to achieve. Under normal circumstances, insulin is delivered from the pancreas into the portal vein. Therefore, intraportal transplantation may provide a more physiologic site for delivery of insulin by transplanted islets. In 1973, Kemp and colleagues demonstrated in a diabetic rat model that intraportal transplantation of islets could successfully and consistently normalize hyperglycemia (47). Numerous other sites of transplantation have been investigated with varied success including intraperitoneal (44, 48-51), intraportal (47, 50, 52-54), intrasplenic (55-57), omental pouch (58-60), renal

subcapsular (55-58, 61-65), thymus (66, 67), testicles (68), ventricles of the brain (69) and bone marrow (70).

The progression from experimental rodent models of islet transplantation to clinical trials of islet transplantation occurred in an astonishingly short period of time during the 1970's. The lack of rigorous large animal models of islet transplantation, including non-human primate models, prior to trials of clinical islet transplantation was controversial (71). However, the lack of large animal and non-human primate models of auto-immune diabetes, then and still presently, raises the question as to whether translation of results from these pre-clinical trials in chemical or surgically induced models of diabetes will accurately reflect the process in an autoimmune diabetic human patient (71).

Several important advances allowed the pioneers of islet transplantation to make this rapid jump from experimental rodent models to clinical trials. A major barrier to human clinical trials was the dense nature of the human pancreas, which prevented adoption of techniques to isolate rodent islets from being used in human islet isolation. The development of a semi-automated method for human islet isolation by Ricordi and colleagues permitted continuous monitoring of enzymatic digestion and collection of increased number of islets (72), while the development of the COBE chamber in 1989 facilitated large-scale Ficoll gradient centrifugation to separate islet from pancreatic exocrine tissue (73).

The first attempt at clinical islet transplantation occurred in 1977 and between 1977 and 1989 fifty-six cases of clinical islet transplantation were described with variable success in attaining transient C-peptide secretion (74). In 1989, Warnock and colleagues

from the University of Alberta reported for the first time the ability of islets from human cadaveric donors to result in sustained C-peptide secretion after a synchronous intraportal islet and orthotopic kidney transplantation (74). Shortly thereafter, Lacy and colleagues at Washington University reported the ability of intraportally transplanted human cadaveric islets to result in insulin-independence in a type 1 diabetic patient (75). These landmark studies answered a key question by demonstrating the ability of human islet allografts to maintain normoglycemia in immunocompromised type 1 diabetic patients.

Consistent and sustained insulin independence remained elusive in clinical islet transplantation that were to follow throughout the 1990's with only 12.4% of 267 type 1 diabetic islet transplant recipients sustaining insulin independence for 1 week and only 8.2% maintaining insulin-independence after 1 year (76). Despite these sobering long-term results, compared with intensive insulin therapy, islet transplantation provided superior metabolic control, prevented hypoglycemic events and held the potential to decrease secondary complications of diabetes (77); a substantial impetus to encourage continued support of the field. Recognition that immunosuppressants can be both toxic to islets and induce peripheral insulin resistance lead to a breakthrough in islet transplantation with the development of the Edmonton Protocol in 2000 (76). The Edmonton Protocol uses an immunosuppressive regimen that avoids the use of corticosteroids while combining sirolimus, tacrolimus, and anti-interleukin-2-receptor antibody therapy to protect the islets against rejection (76). With this immunosuppressive regimen, 100% insulin independence was achieved in 7 consecutive patients with type 1 DM (76).

As of November 2008, the Edmonton group has performed islet transplantations in 108 patients. Long-term results of islet transplantation at the University of Alberta have now been published with 87% of patients maintaining inulin-independence at 1 year. However, using earlier protocols, only 9% of patients remained insulin-independent despite the fact that 80% demonstrated some form of graft function through sustained C-peptide production (78). Using new protocols involving T cell depletion with thyroglobulin and alemtuzumab, intensive heparin therapy, peritransplant insulin administration and islet culture, 100% insulin independence can be maintained up to 2-3 years with 50% of single donor recipients remaining insulin free at 5 years (personal communication Dr. A.M.J. Shaprio). Over 50 sites worldwide have now performed islet transplantation under the Edmonton Protocol (79), with an international multicenter trial demonstrating its feasibility and reproducibility (80). Through this process, however, it has become apparent that superior results in islet transplantation require expertise and experience as not all sites performing islet transplantation are capable of reproducing results of the Edmonton group (81, 82).

1.4.3.1 BARRIERS TO ISLET TRANSPLANTATION

The field of islet transplantation has seen significant advances since the first successful islet transplant in a rodent model by Lacy over 35 years ago (44). While clinical islet transplantation has proven to be an effective treatment for brittle type 1 diabetic patients (76, 78, 80), eventual graft failure in a large proportion of patients (78) has lead some to question the clinical applicability of this treatment for all type 1 diabetic patients (83). Recounting the original vision of Lacy, the goal of islet transplantation

should be to "transplant the islets without immunosuppression early in the course of diabetes" (71). In order to accomplish Lacy's original vision, many challenges must be addressed - most significantly, but not limited to *1)* identifying a source for β cell replacement which will meet the demands of the increasing population of diabetic patients and *2)* preventing islet graft rejection without the need for continuous harmful immunosuppressive drugs.

1.5 PORCINE ISLET XENOTRANSPLANTATION

Current human islet allotransplantation protocols require multiple donors for transplantation and each transplant recipient requires multiple islet transplantations over the course of a lifetime (78). While the use of cadaveric donors increases the donor pool for islets, there remains a critical shortage of islet donors for all potential recipients to be treated. Therefore, a major barrier to the advancement of islet transplantation as a treatment for type 1 DM remains the shortage of islet donors. One near-term solution which has been investigated is the use of porcine islets for the purpose of islet xenotransplantation.

1.5.1 HISTORY

In 1974, Najarian and his colleagues from the University of Minnesota described the first successful attempt at isolating islets from adult pigs (84). Using a technique modified from Lacy's rodent islet isolations, involving duct dilation with cold Hank's solution, collagenase digestion and ficoll gradient separation, Najarian and colleagues were able to successfully isolate intact islets and demonstrate the presence of circulating

insulin following autograft and allograft transplantation in pancreatectomized pigs (84). In 1986, Lacy and his colleagues further refined the process of adult porcine islet isolation by dilating the pancreatic duct with collagenase solution prior to morcellation and ficoll gradient separation (72). Using this technique, a high yield of purified islets were obtained which demonstrated function *in vitro* by glucose stimulation insulin assay and transient *in vivo* function when transplanted into immunosuppressed B6 mice (72). Since this early experience, several significant advances have helped to increase the yield and quality of porcine islet preparations and have also identified alternative sources of porcine islet tissue.

1.5.2 AGE OF PORCINE ISLET DONORS

Porcine islets for transplantation have been derived from 3 sources defined by age of the donor animal: fetal pigs, neonatal pigs and adult pigs. Advantages and disadvantages of each donor source can be identified, however, to date successful large animal islet allotransplantation (59, 85) and non-human primate islet xenotransplantation (86, 87) has only been achieved using neonatal and adult porcine islets.

A simplified protocol to isolate porcine fetal islet-like cell clusters (FICC) was described by Korsgren and colleagues in 1988 (88). This isolation protocol, based on a protocol developed by this same group to isolate human FICC (89), aims to digest the pancreas into small clusters using collagenase and omits the steps of ductal infusion of collagenase and ficoll gradient separation of islet cells. To separate endocrine and exocrine components of the isolate, FICC are cultured for 7 days with every other day media changes (88). While it was noted that insulin secretion was quite uniform, the

functional ability of fetal porcine islets was quite immature and stimulation of islets with glucose and theophylline yielded only minimal amounts of insulin release. Fetal porcine islets, however, differ from adult porcine islets in that they can be maintained in culture for up to 30 days (90) and during this time fetal porcine islets mature resulting in increased insulin secretion (88). Because FICC are functionally immature, they require up to 8 weeks to exhibit function in immune-deficient mice (91). To date, experience in porcine FICC xenotransplantation is limited beyond rodent models. In 1994, the Uppsala group transplanted porcine FICC in the renal subcapsular space of 10 insulin-independent diabetic renal transplant patients (92). While small amounts of porcine C-peptide were detected in the urine of these patients at 200-400 days post-transplantation, none of these patients attained insulin independence.

In 1996, Korbutt and colleagues translated the simplified procedure used for porcine FICC isolation to isolate neonatal porcine islets (NPI) from 1-3 day old pigs with the goal of attaining a more functionally mature islet source (93). Unlike porcine FICC which are unresponsive to glucose stimulation *in vitro* (88), NPI were demonstrated to secrete insulin upon high-concentration glucose stimulation *in vitro* (93). Furthermore, transplantation of 2,000 NPI aggregates under the kidney capsule of an immune-deficient mouse was sufficient to reverse alloxan-induced diabetes (93). However, while post-natal maturation of NPI results in immediate *in vitro* insulin secretion, transplantation of NPI into immune-deficient mice results in delayed reversal of hyperglycemia at 8 weeks post-transplantation (93). The ability of NPI to reverse diabetes has now been demonstrated in a large animal allograft model (59) and a non-human primate model (86). A clinical trial of NPI and Sertoli cell co-transplantation has also been performed in

adolescent patients in Mexico, however, concerns raised by the international community have been raised regarding the selection criteria of these transplant recipients as well as the results of this trial (94).

The benefits of using juvenile or adult pigs as a source of islets for xenotransplantation is that this functionally mature tissue is able to reverse hyperglycemia immediately in rodent and (95) non-human primate models (86, 96). Using a modification of the automated system for human islet isolation, Ricordi and colleagues were able to obtain an average of 255,000 islets per pancreas from 10 to 36 month old pigs with an islet purity of 85-90% (95). Adult porcine islets, however, are more fragile and difficult to maintain in culture for the purposes of decreasing immunogenicity or cryopreservation (95).

At present time, there is no consensus as to the ideal age of pigs to be used for porcine islet xenotransplantation. Islets derived from immature fetal and neonatal pig sources offer several advantages: *1)* they are simpler to isolate possibly facilitating automation of this process, *2)* their isolation is not dependent on collagenase type or lot, *3)* they can be maintained in culture providing the opportunity for post-isolation modification and/or cryopreservation, *4)* and their immature state provides the possibility of sustained proliferation after transplantation (97). The main disadvantage of these immature sources, however, remains the need for a maturational period prior to reversal of diabetes in animal models (86, 91, 93). Adult porcine islet sources, on the other hand, have the advantage of immediately reversing diabetes in animal models (87, 95, 96), however, isolation and culture of adult porcine islets is more difficult given their fragility (95).

1.5.3 CLINICAL APPLICATION OF PORCINE ISLET XENOTRANSPLANTATION

Given the fact that both NPI and adult porcine islets have been demonstrated to function in preclinical non-human primate models (86, 87, 96), it has been suggested that these sources will serve as the donor sources for first iteration of islet xenotransplantation clinical trials (98).

Based on current non-human primate experience, a 70 kg human undergoing islet xenotransplantation will require 3.5×10^6 NPI. Given that the average 1-3 day old neonatal pig yields approximately 50,000 islets (93), approximately 70 neonatal pigs will be required for a single human NPI xenotransplant. In comparison, a human undergoing adult porcine islet xenotransplantation will require 1.5×10^6 islets. A single adult pig may yield 255,000 islets (95), therefore an average 70 kg human recipient will require adult porcine islets from only 6 donors. The shear volume of NPI donors needed to transplant a single human patient would favour an adult porcine islet donor source.

Increased regulatory controls on materials involved in direct human exposure will likely play a large role in selection of a porcine islet source for transplantation. Close control of porcine islet donor herds will be mandated by regulatory boards involved in clinical trials of porcine islet xenotransplantation and will involve donor animals being designated pathogen-free (99). Breeding colonies can be established by early weaning or delivery by caesarian derivation into a pathogen-free housing facility and subsequent testing for pathogen free status. Current recommendations are for animals to be housed for 2 generations prior to being released as sources for clinical transplantation (99). Once born, islet donors will be maintained in pathogen-free conditions in compliance with

Good Manufacturing Practice (GMP), fed pathogen free food and water and thoroughly tested for bacterial, parasitic and viral pathogens (99). Therefore, while utilization of NPI as an islet donor source would necessitate the use of large numbers of islet donors, only breeding pairs would be maintained under GMP conditions while donor animals would be processed for islet transplantation soon after birth. On the other hand, while only 6 adult porcine islet donors would be required for a single human islet recipient, all 6 donor animals would need to be housed and tested under GMP conditions for 10 to 36 months prior to transplantation which may escalate costs to prohibitive levels. Regardless of the source of porcine islet donor chosen, if clinical islet xenotransplantation is to succeed, automated means of isolating porcine islets will need to be developed to provide adequate donor islets to meet the volume of diabetic patients around the world.

1.5.4 INNATE IMMUNE MEDIATED ISLET XENOGRAFT REJECTION

1.5.4.1 INSTANT BLOOD MEDIATED INFLAMMATORY REACTION

Both allogeneic and xenogeneic islet transplants when exposed directly to blood, such as the case with portal vein transplantation, undergo significant and rapid tissue loss (100, 101). Exposure of islets to blood induces a rapid thrombotic/inflammatory reaction termed Instant Blood Mediated Inflammatory Reaction (IBMIR), which is thought to account for the rapid and significant tissue loss seen in clinical islet transplantation. First described by Korsgren and colleagues, IBMIR is the result of rapid activation of the coagulation and complement systems, recruitment and infiltration of neutrophils, and activation of platelets resulting in intravascular thrombus formation and resultant islet destruction (100-102). Membrane bound tissue factor on islets and an alternatively

spliced form released in association with insulin and glucagons has been demonstrated to be a key component in initiating IBMIR (103). Several interventions have been demonstrated to prevent IBMIR including direct inhibition of tissue factor with recombinant FVIIa (104), heparin and soluble complement (101) and low molecular weight heparin (105).

1.5.4.2 ANTIBODY-MEDIATED HYPERACUTE REJECTION

The presence of naturally occurring preformed antibodies is another potential barrier to initial islet engraftment in recipients. In porcine islet xenotransplantation, recipients such as humans, apes and old world monkeys possess preformed antibodies to galactosyl alpha (1–3) galactose (αGal) - a carbohydrate moiety found on many mammalian cells (106). While αGal expression is very high on endothelial tissues making antibody-mediated hyperacute rejection a formidable concern in vascularized xenografts (107), the extent antibody-mediated hyperacute rejection to αGal in islet xenotransplantation is less clear. The expression of αGal on porcine β cells is related to islet maturity, with FICC and NPI expressing αGal, however, as β cells mature αGal expression becomes negligible (106, 108). Despite the lack of αGal expression on adult porcine islet cells, some studies have suggested that αGal expression does affect islet immunogenicity. Komoda and colleagues have developed a transgenic pig that overexpresses N-acetylglucosaminyltransferase-III (GnT-III), an enzyme which competitively glycosylates trans-golgi carbohydrates and prevents the formation of αGal (109). When adult porcine islets from this transgenic model were transplanted into streptozotocin-induced diabetic monkeys, it was found that islets from the GnT-III

transgenic pig did not undergo hyperacute rejection and showed less humoral and cellular rejection (110).

1.5.4.3 INNATE IMMUNE CELL REJECTION

The role of innate immune cells in the rejection of porcine islet xenografts at current time is incompletely defined. Examining the immune reaction associated with fetal porcine islet xenografts in murine models, numerous types of innate immune cells have been temporally associated with rejecting xenografts including macrophage, NK cells and eosinophils (111-113). While eosinophils are temporally associated with fetal porcine islet xenograft rejection, in the absence of eosinophils islet xenograft rejection kinetics are unaffected suggesting a negligible role of these innate immune cells in the rejection process (113). Other innate immune cells - such as macrophage and NK cells - on the other hand, play a more important role.

Several studies have demonstrated that macrophage depletion results in prolongation of porcine islet xenograft survival when transplanted under the renal capsule (114), intraportally (115) and also intraperitoneal transplantation of microencapsulated islets (116). It appears that macrophage can act as effector cell that carries out porcine islet rejection. Yi and colleagues demonstrated that if $CD4^+$ T cell-activated macrophage were transferred to NOD-SCID mice, they were capable of rejecting fetal porcine islet xenografts (117). In contrast, when unactivated macrophage were transferred in this model, islet xenografts were not rejected (117).

The role of NK cells in islet xenograft rejection, on the other hand is less clear. Our group has shown that NK cell depletion using the monoclonal antibody (mAb) NK

1.1 in a concordant xenograft model - where rat islets are transplanted to B6 mice - prolongs graft survival (Chapter 3). However, after administering anti-NK 1.1 mAb in a discordant adult porcine islet xenotransplant model, Wu and colleagues did not find a significant prolongation in porcine graft survival in mice (114). Currently, it is unknown what role NK cells play specifically in the rejection of NPI xenografts. Additionally, application of results from discordant models of xenotransplantation is confounded by the fact that clinical porcine islet xenotransplantation represents a concordant model of xenotransplantation.

1.5.5 T CELL MEDIATED REJECTION

T cell mediated rejection of porcine islets represents a major barrier to xenograft survival and several studies in small-animal models using non-depleting anti-CD4 monoclonal antibodies (118), depleting anti-CD4 monoclonal antibodies (119) and $CD4^+$ T cell knock-out mice (120) have demonstrated the importance of specifically $CD4^+$ T cells in this rejection process. T cell activation occurs through a two-signal process. Signal 1 is an antigen dependent process involving the engagement of a major histocompatibility complex II (MHC II) bound antigen with a T cell receptor (TCR)/CD3 complex. Signal 2, on the other hand, is antigen independent and involves the interaction of costimulatory ligands and their corresponding receptors expressed on T cells and antigen presenting cells (APC). With respect to T cell activation in transplantation immunology, this process can occur by two means: direct and indirect T cell activation. Direct T cell activation involves the presentation of transplant antigens by donor APC to the TCR/CD3 complex of host T cells. Indirect T cell activation,

meanwhile, requires transplant antigens to be processed and presented by host APC and subsequently presented to host T cells. Islet allograft rejection involves both the direct and indirect antigen presentation pathways, with the direct pathway being the dominant route of T cell activation (121). The dominant method of T cell activation in islet xenograft rejection, conversely, is the indirect T cell activation pathway (122, 123). The degree of dependence on the indirect T cell activation pathway, however, is related to the phylogenetic disparity between donor islet source and host species (122). The relatively close combination of rat islets transplanted into mouse recipients, while dependent on MHC II-dependent indirect antigen presentation, can ultimately result in xenograft rejection through the direct antigen presentation pathway (122). The relatively disparate combination of porcine islets transplanted into mouse recipients, meanwhile, is completely dependent on the indirect pathway as MHC class II – deficient recipient mice are unable to reject their porcine islet xenografts indefinitely (122). These findings demonstrate that $CD4^+$ T cell activation by the indirect pathway is absolutely essential in porcine islet xenograft rejection and consequently anti-rejection therapies to date have focused on preventing this interaction from occurring.

While $CD4^+$ T cells play a central role in porcine islet xenograft rejection, $CD8^+$ T cells may play a minor role in rejection in the presence of $CD4^+$ T cells. Koulmanda and colleagues found that the depletion of $CD8^+$ T cells in the presence of $CD4^+$ T cells prolonged the survival of fetal porcine islet xenografts, however, islet xenografts were ultimately rejected. Moreover, subsequent depletion of $CD4^+$ T cells resulted in long-term graft survival (123). These results provided further evidence that porcine islet

xenograft rejection relies predominantly on $CD4^+$ T cells while $CD8^+$ T cells are only minimally involved in xenograft rejection.

1.5.6 ANTIBODY MEDIATED REJECTION

If hyperacute and acute cellular rejection mechanisms are overcome, another potential barrier to porcine islet xenotransplantation could be the generation of non-Gal antibodies in response to $CD4^+$ T cell activation of B lymphocyte responses. Our group has found that in untreated mice transplanted with NPI, an anti-porcine humoral response is generated at day 5 post-transplantation and antibody levels increases until time of rejection at 7 days (Chapter 2). McKenzie and colleagues demonstrated that administration of CD46, a complement regulator, could prevent antibody mediated rejection of fetal porcine islets in SCID mice (124).

1.5.7 PIG ENDOGENOUS RETROVIRUS

In the past decade concern over latent, endogenous infectious agents has been raised in the use of porcine tissue for xenotransplantation. In particular, Porcine Endogenous Retrovirus (PERV) is a C-type retrovirus which is incorporated into the porcine genome. Three subtypes of PERV exist, classified based on the *env* subfamily expressed within the virus (Figure 1-2). PERV A and B is found in the genome of all pigs while some strains of pigs lack PERV C (125).

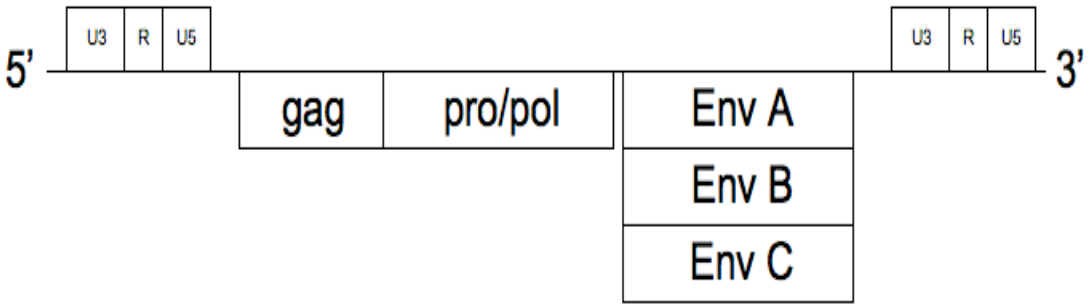

Figure 1-2: Organization of the porcine endogenous retrovirus genome. Three subtypes of PERV exist classified by the *env* gene subtype. (Adapted from Blusch, et. al., Xenotransplantation 2002, 9:242-251)

There are currently three accepted approaches for detecting PERV infectivity. *1)* detection of PERV DNA sequences via PCR, *2)* detection of PERV RNA sequences via RT-PCR and *3)* immunologic assays detecting anti-viral antibody production using western blot analysis or ELISA (125). Detection of PERV DNA sequences is confounded by the presence of endogenous PERV sequences within porcine tissue samples. The use of real-time PCR improves sensitivity of this approach as PERV DNA sequences can be measured against porcine mitochondrial DNA sequences to rule out the presence of porcine tissue within a sample (125). Alternatively, RT-PCR may be used to detect transcribed PERV RNA sequences found in cells, infected tissue or packaged into viruses. However, expression of PERV RNA does not necessarily signal the release of viral particles as the majority of PERV loci within the pig genome contain frameshift mutations and stop codons preventing full transcription of the entire viral sequence (125). Lastly, detection of anti-viral antibody production with various immunologic approaches is the most sensitive method for detecting ongoing PERV replication and infectivity. However, major drawbacks with immunological approaches are that other retroviral

infections may result in false-positive tests and any anti-rejection therapies which down-regulate B cell responses may also blunt the anti-viral antibody response (125).

Evidence of PERV infecting human cells *in vitro* was first brought to light in 1997 (126). While it is not known what triggers expression of the retrovirus, *in vitro* (127) and *in vivo* (128, 129) work suggests that mitogenic and post-operative hypoxic stress may trigger the expression of PERV. Despite experimental data to suggest the possibility of PERV transmission to xenotransplant recipients, retrospective reviews of human patients exposed to porcine tissue (130) and specifically porcine islets (131) (132) have not identified cases of transplant recipients infected long-term with PERV. While current evidence suggests that PERV infection does not occur *in vivo* in human host cells, in this era of concern regarding zoonotic infections such as the avian flu and Creutzfeldt-Jakob disease, sensitive yet accurate testing for PERV is paramount as both true-positive and false-positive results of PERV infection *in vivo* would have a crippling effect on efforts at porcine islet xenotransplantation.

1.5.8 LARGE ANIMAL TRIALS

While much information has been gleaned from rodent models of islet xenotransplantation, their applicability to human subjects is often in question. In 1992, Tollemar and colleagues demonstrated the safety of intra-portal injection of porcine islet-like cell clusters in a canine model (133). Since that time, however, clinically applicable porcine islet xenotransplant models have been performed primarily in non-human primates. Despite the vast resources and expenses necessary, there is great interest in non-human primate models of porcine islet xenotransplantation as they likely represent a

closer physiologic environment to that seen in humans. A major limiting factor, however, is the lack of a non-human primate model of autoimmune diabetes.

Soderlund and colleagues demonstrated that fetal porcine islets transplanted under the renal sub-capsule of cynomolgus monkeys underwent acute cellular rejection (134). When immunosuppressed with cyclosporine and 15-deoxyspergualin (DSG), cellular infiltration was delayed but ultimately the graft underwent rejection by day 15. This model confirmed that similar to small animal models, porcine islet xenotransplantation in primates also undergo cellular rejection. Nevertheless, focus has shifted to models that utilize portal vein injection of islets to more closely approximate the clinical model of islet transplantation.

Portal vein transplantation models in non-human primates demonstrated that porcine islet xenotransplantation was feasible but were also acutely rejected despite the use of powerful immunosuppressant regimes (135, 136). Both studies, however, lacked specific treatment to guard against IBMIR and likely reflect a rejection mechanism not seen in small animal models. Despite the fact that xenografts were rejected acutely after only 1-2 days, they did transiently result in detectable C-peptide levels (135, 136).

In 2004, Hering and colleagues successfully transplanted adult porcine islets intraportally into rhesus macaques and were able to achieve euglycemia (137). When injected intraportally, these transplants were not subjected to hyperacute rejection, putting into question the role of αGal mediated hyperacute rejection. Beginning at 24 hours post-transplantation, however, these xenografts displayed evidence of cellular infiltration and subsequently were rejected with the infiltrate characterized by the presence of $CD4^+$ T cells, $CD8^+$ T cells and macrophage. More recently, the Edmonton/Atlanta and

Minnesota groups simultaneously demonstrated long-term success in transplanting NPI and adult porcine islets, respectively, into non-human primates (86, 87). Cardona and colleagues were able to induce sustained insulin independence for a median of >140 days in rhesus macaques transplanted with NPI using an immunosuppression regime of anti-IL2 receptor mAb, anti-CD154 mAb and maintenance immunosuppression of sirolimus and belatacept (86). Meanwhile, Hering's group using a slightly different immunosuppression protocol of induction basiliximab and FTY720 with maintenance immunosuppression of anti-CD154 mAb, everolimus (sirolimus derivative) and leflunomide, were able to prolong adult porcine islet xenograft survival for over 1000 days in 4 of 5 recipients (87). For the first time, these simultaneous studies demonstrated that porcine islet xenograft function could be sustained long-term in a preclinical model. In immunosuppressed non-human primates, immune cell infiltration of the islet grafts was suppressed (86, 87) as was the production of non-Gal porcine IgG (87). Importantly, it was also demonstrated that over the length of the study there was no evidence of PERV transmission from NPI to the recipients (86).

1.5.9 HUMAN CLINICAL TRIALS

The first published porcine islet xenotransplant trials were performed by Groth and colleagues in 1990 (92). Fetal porcine islets were transplanted in 10 insulin-dependent diabetics who had previously or were undergoing renal transplantation. The fetal porcine islets were transplanted either via intraportal injection or under the kidney capsule. This pilot study demonstrated that islet grafts injected neither intraportally nor under the renal capsule resulted in immediate complications. However, 4 of 10 patients

did excrete porcine C-peptide several months post-transplant suggesting that hyperacute reaction did not occur and that survival of porcine islet xenografts is possible in human recipients.

More recently, another human clinical trial was performed in Mexico by Valdes and his colleagues (94). Using a vascularized collagen sheath implanted subcutaneously, type I diabetic adolescents were co-transplanted with NPI and Sertoli cells. Without immunosuppression it was reported that 50% of the transplanted patients saw a significant reduction in their insulin requirements and 2 of the 12 transplanted patients were transiently free of insulin injections. There has been much controversy in the xenotransplantation community regarding this trial and whether adequate large animal trials have been conducted prior to the commencement of islet xenotransplantation in humans.

1.6 FUTURE POTENTIAL THERAPIES

Several alternative approaches to delivering insulin in response to fluctuating glucose levels have been attempted with varied success which include *1)* bioartificial pancreases, *2)* gene therapy, *3)* stem cells and *4)* β cell regeneration. Closed-loop bioartificial pancreases are composed of three components: a glucose sensor, an insulin pump and a stable computer algorithm to control insulin delivery (138). Using current technology, glucose sensors can detect blood glucose levels through enzymatic reactions, however, require chronic implantation of a probe for direct blood sampling. Alternatively, optical sensors can also detect blood glucose levels, however, this technology is limited by interference from other soluble components in blood (138).

While the technology exists to produce compact and efficient insulin delivery systems, limits in real-time blood glucose level detection are the main hindrances in the advancement of closed-loop bioartificial pancreases as a treatment option for type 1 DM patients (138).

Gene therapy has been proposed as a treatment for type 1 DM. In order to evade β cell specific autoimmune attack, introduction of the insulin gene into non-β cells would theoretically result in subsequent autoimmune destruction of insulin producing cells. Despite the fact that expression of biologically active transgenic insulin has been achieved (139), this strategy is currently limited by the inability to transfer to non-β cells complex glucose-sensing mechanisms required to secrete insulin within a narrow physiologic range (139).

The ability to generate de novo β cells through differentiation of pluripotent stem cells remains an attractive option as a future therapy for type 1 DM patients. Two potential sources of stem cells exist, embryonic stem cells (ESC) and mesenchymal stem cells (MSC). ESC have the potential to differentiate into insulin producing β cells if guided along appropriate developmental pathways (140). Two transcription factors, Pdx-1 and neurogenin-3, have been identified as important factors in the differentiation of ESC into islet cells (141). In diabetic mouse models, the ability to differentiate ESC into insulin producing β cells with the ability to reverse hyperglycemia has already been demonstrated (142, 143). A major hurdle in ESC research, however, are the ethical barriers surrounding ESC line procurement. MSC are derived from adult bone marrow, fat and several fetal tissues (144). Under appropriate environments, MSC are capable of differentiating into mesodermal, endodermal and ectodermal cells – including β cells.

Several groups have demonstrated that nestin-positive pancreatic ductal progenitor cells are capable of differentiating into insulin-expressing cells both *in vitro* (145) and *in vivo* (146, 147) with the ability to reverse hyperglycemia in diabetic mice (148). The use of pluripotent stem cells to generate insulin producing β cells offers an ambitious and promising solution to the shortage of human islet donors, however, this field of research remains in its infancy and a clinical solution to type 1 DM using this approach appears to be many years away.

The ability for the β cells to regenerate has recently been recognized by the scientific community. With this understanding, a fourth approach to the treatment of type 1 DM is to develop methods of regenerating and increasing β cell mass to the point of restoring euglycemia. Certainly a challenge in applying β cell regeneration to the treatment of type 1 DM patients is the ongoing presence of autoimmunity. However, when autoimmunity is blocked, it has been demonstrated in humans (149-151) and rodents (152, 153) that β cell function can partly recover. Using genetic lineage tracing, it has been demonstrated that regeneration of differentiated β cells, rather than differentiation of stem cells is the reason for β cell recovery (154). While the innate ability of β cells to regenerate, even after autoimmune damage, is an extremely attractive approach toward treating type 1 DM, it is currently unclear whether autoimmunity against β cells can be adequately over come in type 1DM.

The goal of future strategies to treat type 1 DM is to provide an unlimited source of glucose responsive insulin production while avoiding the need for immunosuppression currently required in islet cell transplantation protocols. Many of these new techniques are still in their developmental infancy and it appears that transplantation of xenogeneic

sources of islets may provide a near-term solution to the shortage of islet donors. Furthermore, if the development of tolerogenic anti-rejection regimes can prove to be successful, islet cell xenotransplantation may prove to be a highly effective treatment for all diabetic patients.

1.7 OBJECTIVES AND GENERAL OUTLINE

The main objective of this thesis was to examine the mechanisms of rejection of NPI xenograft rejection. Three areas of NPI xenograft rejection were assessed: 1) The immune cells and molecules involved in the early events of NPI xenograft rejection, 2) The role of NK cells in both concordant rat and discordant NPI xenograft rejection, and 3) The effects of T-cell co-stimulatory and adhesion pathway blockade on the rejection of NPI xenografts.

The early events of NPI xenograft rejection in a non-autoimmune, immune competent B6 mouse model is examined in chapter 2. We investigated local graft site events during NPI xenograft rejection examining the immune cells present within the graft site using immunohistochemical staining and also examining the chemokines and cytokines produced at the graft site using reverse-transcriptase polymerase chain reaction. Early systemic changes in the recipient mice were investigated using flow cytometry to examine immune cell populations and the production of anti-porcine IgG antibody production while a multiplex bead immunoassay was used to measure the systemic production of chemokines and cytokines. In chapter 3, we compared the role of NK cells in a concordant model of islet xenograft rejection using rat islets to a discordant model using NPI xenografts in a non-autoimmune, diabetic mouse model. In chapter 4, we

examined the effects on NPI xenograft survival of blocking T cell co-stimulatory pathways and adhesion pathways both alone and in combination with each other.

Islet transplantation is a potentially curative treatment for type 1 DM. Unlike insulin therapy, islet transplantation has the potential to regulate BG levels more physiologically and may potentially prevent secondary complications of DM. Unlike pancreatic transplantation, however, islet transplantation is relatively less invasive and less morbid. At present, two major barriers limit the number of diabetic patients able to benefit from this treatment – the shortage of human islet donors and the need for continuous immunosuppressive therapy. NPI xenotransplantation provides a potentially unlimited source of islets for transplantation. Efforts to understand the mechanisms of rejection of NPI may unlock the key to islet xenotransplantation without the need for continuous immunosuppressive therapy.

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CHAPTER TWO

IMMUNE CELLS AND MOLECULES INVOLVED IN THE EARLY EVENTS OF NEONATAL PORCINE ISLET XENOGRAFT REJECTION

2.1 INTRODUCTION

Islet transplantation is an attractive alternative treatment for type 1 diabetic patients. However, two major hurdles stand in the way of islet transplantation gaining widespread acceptance; the limited supply of islets and the use of harmful immunosuppressive drug regimes. One strategy to overcome the shortage of human islets for clinical transplantation is through the use of xenogeneic islets, in particular neonatal porcine islets (NPI). Transplantation of 2,000 NPI under the kidney capsule of alloxan-induced diabetic nude mice can correct hyperglycemia in 100% of recipients within 8 weeks post-transplantation (1). Recently, it has been demonstrated that NPI can also reverse hyperglycemia in pre-clinical non-human primate models (2, 3). Therefore in the near-term, it appears that NPI xenografts may be a feasible option to provide a potentially unlimited source of islets for clinical islet transplantation.

However for progress in NPI xenotransplantation to continue, preventing the immunologic barrier to islet xenotransplants requires further refinement. A better understanding of NPI xenograft rejection processes will facilitate the identification of new targets for novel anti-rejection therapy and enable more specific targeting of the immune cells and molecules involved. We have previously demonstrated that rejection of NPI xenografts transplanted under the kidney capsule of rodents is highly dependent on $CD4^+$ T cells and the indirect pathway of antigen recognition (4). While the central role of $CD4^+$ T cells in NPI xenograft rejection is now well recognized, it is currently unclear how $CD4^+$ T cells interact with the remainder of the immune system and direct islet cell killing.

In this study, we examined early events of NPI xenograft rejection in the absence of auto-immunity in an immune-competent B6 mouse transplant model examining both the local graft site immune cells and molecules involved in rejection and the systemic events in response to a rejecting NPI xenograft. Our data demonstrate that NPI transplanted into B6 mice are acutely rejected by day 7 post-transplantation. The innate immune cells, macrophage and neutrophils, are the first immune cells present at the graft site on day 2 post-transplantation. Meanwhile, $CD4^+$ T cells, B cells and eosinophils were detected on day 4 and $CD8^+$ T cells were observed on day 5 post-transplantation. Several chemokines and cytokines were expressed within the graft site during the course of rejection with MIP-1β being produced specifically in response to NPI xenografts. In contrast, systemic immune cell populations, chemokines and cytokines do not change in the early stages of NPI xenograft rejection. Therefore, early events in NPI xenograft rejection appear to be localized to the graft site and therapies aimed at targeting this localized response may result fewer systemic side effects making islet xenotransplantation a more attractive option for diabetic patients.

2.2 MATERIALS AND METHODS

2.2.1 ANIMALS

One to 3 day old Landrace-Yorkshire neonatal pigs of either sex (1.5-2.0 kg, University of Alberta farm, Edmonton, AB, Canada) served as islet donors and 6-8 week old male C57BL/6J (B6, H-2^b, Jackson Laboratory, Bar Harbor, ME, USA) mice served as recipients. All animals were fed standard laboratory food and cared for according to

the guidelines established by the Health Sciences Animal Policy and Welfare Committee at the University of Alberta.

2.2.2 ISLET ISOLATION

NPI were isolated as previously described (1). Briefly, neonatal pigs were anesthetized and subjected to laparotomy and exsanguinated by means of abdominal aortic catheterization. The pancreas was surgically removed under sterile conditions and placed in cold Hank's Buffer Salt Solution (HBSS, Sigma, St Louis, MO, USA). Sterile scissors were used to cut the pancreas into 1-2 mm pieces and then digested with 2.5 mg/ml collagenase (Sigma). Digested tissue was filtered through a 500 μm nylon screen then cultured for 7 days in Ham's F10 medium containing 10 mmol/l glucose, 50 μmol/l isobutylmethylxanthine (ICN Biomedicals, Montreal, PQ, Canada), 0.5% bovine serum albumin (fraction V, radioimmunoassay grade; Sigma), 2 mmol/l L-glutamine, 3 mmol/l $CaCl_2$, 10 mmol/l nicotinamide (BDH Biochemical, Poole, England), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C, 5% CO_2, 95% air. A complete media change was performed on culture days 1, 2, 4 and 6.

2.2.3 EXPERIMENTAL DESIGN

After 7 days of culture, islets were transplanted under the left kidney capsule of naïve B6 mice as previously described (1). Briefly, non-diabetic, naïve B6 mice were anesthetized by inhalational isoflurane and the left flank was shaved and sterilized with 100% ethanol. A flank incision was made lateral to the left paraspinal muscles and the peritoneum was sharply divided to expose the left kidney. A sterile cotton swab was

utilized to expose and produce the left kidney from the incision. A small incision was made in the kidney capsule with a 27 gauge needle and a subcapsular pocket was expanded with the use of a sterile Pasteur pipette. Aliquots of 2,000 NPI were aspirated into polyethylene (PE-50) tubing, pelleted by centrifugation and placed within the subcapsular pocket with the aid of a micromanipulator syringe. Once the tubing was removed, the kidney capsule was cauterized with a disposable high-temperature cautery pen (Aaron Medical Industries, St. Petersburg, FL, USA). In sham operation control mice, an equivalent volume of HBSS was placed within the subcapsular pocket.

2.2.4 IMMUNOHISTOLOGICAL ANALYSIS

Graft-bearing kidneys and sham operated kidneys were collected at 6h, 12h, 1d, 2d, 3d, 4d, 5d, 6d and 7d post-transplantation. Specimens for immunohistological insulin and eosinophil analysis were fixed with 10% buffered formalin solution then embedded in paraffin. Tissue sections of 5 μm thickness were incubated with guinea pig anti-insulin antibody (1:1000, Dako Laboratories, Mississauga, ON, Canada) or rabbit anti-mouse eosinophil antibody (1:500, kindly provided by Dr. Moqbel, University of Alberta) for 30 min followed by an incubation with biotinylated goat anti-guinea pig IgG secondary antibody (1:200, Vector Laboratories, Burlington, CA, USA) or anti-rabbit secondary antibody (1:200 Vector Laboratories) for 20 min. Avidin-biotin complex/horseradish peroxidase (ABC/HP, Vector Laboratories) and 3, 3-diaminobenzidinetetrahydrochloride (DAB, BioGenex, San Ramon, CA, USA) were applied to tissue sections to produce a brown color to positively stained cells. Tissue sections stained for insulin were counterstained with Harris' hematoxylin and eosin to

visualize the infiltrating immune cells in the graft whereas sections stained for eosinophils were counterstained with Harris' hematoxylin alone.

Concurrently, specimens for immunohistochemical analysis of immune cells within the graft were immersed in optimal cutting temperature (OCT) compound (Ted Pella Inc, Redding, CA, USA) and snap frozen at -80° C. Five μm thick cryopreserved sections of tissue were air dried for 10 min then fixed in acetone for 3 min at 4° C. Cryopreserved sections were then washed in PBS and subsequently incubated in 2% FBS/PBS for 20 min to avoid non-specific binding. Endogenous biotin or biotin-binding proteins within the sample were blocked with an avidin/biotin blocking kit (Vector Laboratories). Rat anti-mouse CD4 antibody (1:100, BD Pharmingen, Mississauga, ON, Canada), rat anti-mouse CD8 antibody (1:100, BD Pharmingen), rat anti-mouse CD11b antibody (1:500, BD Pharmingen), rat ant-mouse CD19 antibody (1:200, BD Pharmingen) or rat anti-mouse neutrophil antibody (1:250, HyCult Biotechnology, Uden, Netherlands) was applied to each tissue sample for 30 min at room temperature. Subsequently, biotinylated rabbit anti-rat IgG secondary antibody (1:200, Vector Laboratories) was applied for 20 min at room temperature. Staining was developed with ABC/HP and DAB as previously described and cryopreserved sections were counter-stained with Harris' hematoxylin.

2.2.5 DETECTION OF MOUSE ANTI-PORCINE ANTIBODIES

Peripheral blood samples were collected from recipient mice at 6h, 12h, 1d, 2d, 3d, 4d, 5d, 6d and 7d post-transplantation as well as from naïve B6 mice. Blood was allowed to coagulate for 30 min at room temperature and centrifuged at 10,000 rpm for

10 min to isolate serum. Spleen cells (1 x 10^6) from donor Landrace-Yorkshire piglets were incubated in 1 ml of a 1:128 dilution of serum from recipient mice transplanted with NPI for 1 hour at 37˚C (5% CO_2, 95% air). Spleen cells were then washed with 1x PBS and incubated with a 1:200 dilution of FITC-conjugated rat adsorbed goat anti-mouse IgG (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) for 1 hour at 4 ˚C. Spleen cells were then washed twice in 1x PBS and resuspended in 500 µl of FACS buffer (1x PBS, 2% FBS). The percentage of NPI spleen cells bound to mouse antibody was determined from fluorescence histograms on a BD FACS Calibur flow cytometry machine (BD Biosciences, Mississauga, ON, Canada) after gating on viable lymphocytes. Control experiments included donor spleen cells alone or donor spleen cells incubated with recipient mouse serum without secondary antibody.

2.2.6 RNA EXTRACTION AND REVERSE-TRANSCRIPTASE PCR ANALYSIS

To identify the immune molecules at early events of NPI xenotransplantation rejection, graft-bearing kidneys and sham operated kidneys were collected at 6h, 12h, 1d, 2d, 3d, 4d, 5d, 6d and 7d post-transplantation to generate cDNA by reverse transcription (RT) and further processed for polymerase chain reaction (PCR) analysis. Islet grafts and surrounding kidney parenchyma were collected using sterile instruments treated overnight with RNAse Away (Molecular Bio-Products, San Diego, CA, USA). In sham operated controls, a segment of kidney parenchyma underlying the area of manipulation was collected in a similar fashion. Naïve B6 mouse kidney, thymus, lymph node and spleen tissue was also collected as control tissue for RNA extraction and subsequent RT-PCR analysis. Tissue samples were immediately pulverized by hand with sterile, RNAse

Away treated pestels, resuspended in Trizol (Sigma) and frozen at -80° C. cDNA was constructed from 1 μg of total RNA using Superscript RNAse H- Reverse Transcriptase (Invitrogen, Burlington, ON, Canada) according to the manufacturer's protocol and 1 μl of cDNA was amplified for 35 cycles using Taq DNA Polymerase (Invitrogen). The PCR conditions were as follows: 30 sec denaturation at 94° C, 30 sec annealing at 58° C, 30 sec extension at 72° C and a final extension at 72° C for 10 min followed by a 4° C hold. Products were separated on an ethidium bromide-stained 2% agarose gel and images were captured with Alpha Digidoc software (Perkin-Elmer, Boston, MA, USA). Primer sequences for MCP-1 (249 bp; accession no. NM_011333), MIP-1α (224 bp; accession no. M73061), MIP-1β (175 bp; accession no. X62502), RANTES (494 bp; accession no. NM_013653), IFNγ (504 bp; accession no. XM_125899), IL-4 (400 bp; accession no. NM_021283), mTGFβ1 (269 bp; accession no. NM_011577), IL10 (406 bp; accession no. NM_010548) and GAPDH (310 bp; accession no. NM_008084) were created based on sequences in Table 2-1. Positive controls consisted of mouse thymus and lymph node cDNA while negative control consisted of water in place of experimental cDNA. GAPDH primers (housekeeping gene) insured cDNA integrity and all primer pairs spanned at least one intron insuring no genomic DNA was detected during amplification.

2.2.7 DETERMINATION OF SYSTEMIC T AND B CELL POPULATIONS

T and B cell populations from islet recipient B6 mice were analyzed and compared with T and B cell populations in sham operated control B6 mice. One million spleen cells were incubated with 100 μl of a 1:100 dilution of the following mAbs: 1) PE-

conjugated anti-CD4/Cy^5-conjugated anti-TCR β-chain (BD Pharmingen), 2) PE-conjugated anti-CD8/Cy^5-conjugated anti-TCR β-chain (BD Pharmingen), and 3) PE-conjugated anti-CD19 (BD Pharmingen), at 4˚C for 30 minutes. Spleen cells were washed two times with PBS and suspended in 500 μl of FACS buffer (2% FBS in 1x PBS). Fluorescence histograms were created using a BD FACS Calibur flow cytometer (BD Biosciences, Mississauga, ON, Canada) and were used to determine the percentage of cells that expressed CD4, CD8, and CD19. Controls for this experiment included unstained spleen cells from islet recipient B6 mice and sham operated control B6 mice.

Table 2-1: RT-PCR primer sequences for MCP-1, MIP-1α, MIP-1β, RANTES, IFNγ, IL-4, mTGFβ1, IL10 and GAPDH

Molecule	Forward sequence (5' → 3')	Reverse sequence (5' → 3')
MCP-1	AGGTCCCTGTCATGCTTCTG	TCTGGACCCATTCCTTCTTG
MIP-1α	TGCCCTTGCTGTTCTTCTCT	CCCAGGTCTCTTTGGAGTCA
MIP-1β	GCCCTCTCTCTCCTCTTGCT	ATACCACAGCTGGCTTGGAG
RANTES	GTGCCCACGTCAAGGAGTAT	GGGAAGCGTATACAGGGTCA
IFNγ	AGCTCTGAGACAATGAACGC	GGACAATCTCTTCCCCACCC
IL-4	TCTTTCTCGAATGTACCAGG	CATGGTGGCTCAGTACTACG
mTGFβ1	TGAGTGGCTGTCTTTTGACG	TTGGTATCCAGGGCTCTCC
IL10	CAAACAAAGGACCAGCTGGAC	GAGTCCAGCAGACTCAATAC
GAPDH	AATCCCATCACCATCTTCCA	GGCAGTGATGGCATGGACTG

2.2.8 DETECTION OF NATURAL KILLER CELLS IN THE PERIPHERAL BLOOD

Peripheral blood from islet recipient and sham operated control B6 mice was collected by cardiac puncture and anti-coagulated by mixing with 500 i.u. of heparin

(LEO Pharma Inc., Thornhill, ON, Canada). Mononuclear cells were isolated by lympholyte mammal (Cedarlane, Burlington, ON, Canada) gradient separation. Subsequently, 5 x 10^5 peripheral blood mononuclear cells (PBMCs) were incubated for 30 min at 4˚C with FITC-conjugated anti-CD49b/Pan-NK cell mAb diluted 1:100 (DX5, BD Biosciences Pharmingen, Mississauga, ON, Canada) then washed two times with 1x PBS. Data were acquired using a BD FACS Calibur flow cytometer (BD Biosciences, Mississauga, ON, Canada). Viable lymphocytes were gated and the proportion of CD49b expressing cells was determined.

2.2.9 DETECTION OF SYSTEMIC CHEMOKINE AND CYTOKINE LEVELS

Systemic levels of chemokines and cytokines were detected in the serum of B6 mice using a multiplex bead immunoassay kit (Biosource, Camarillo, CA, USA) following the manufacturer's instructions. Briefly, peripheral blood samples were collected from recipient and sham operated control B6 mice at 2d, 4d, 5d and 7d post-transplantation, allowed to coagulate at room temperature for 30 min and centrifuged at 10,000 rpm for 10 min to isolate the serum. This assay employs a solid phase sandwich technique to measure analyte concentration. Fifty μl of serum and standard concentration analytes were incubated with beads of defined spectral properties conjugated to specific capture antibodies to chemokines and cytokines. Beads and captured analytes were then incubated with analyte-specific biotinylated detector antibodies. Finally, beads were incubated with a fluorescent protein, R-Phycoerythrin, conjugated to Streptavidin. Beads were then analyzed with a Luminex 100 instrument (Luminex Corporation, Austin, TX, USA) which identifies specific analytes based on the spectral properties of the bound

beads as well as the concentration of each analyte based on the emitted fluorescence as compared to a standard curve generated from known analyte concentrations.

2.3 RESULTS

2.3.1 NEONATAL PORCINE ISLET XENOGRAFT REJECTION IS CHARACTERIZED BY EARLY INNATE IMMUNE CELL INFILTRATION FOLLOWED BY ADAPTIVE IMMUNE CELL INFILTATION

Neonatal porcine islet xenografts were collected at various time points post-transplantation to examine the immune cells involved in the rejection process. An abundance of insulin positive staining β cells are present in the early post-transplantation period from 6 hours to 2 days. However, while the number of infiltrating immune cells increase beginning on 2 days post-transplantation, insulin positive β cells are detected until 5 days post-transplantation. By 7 days post-transplantation there is no further evidence of surviving β cells within the graft (Figure 2-1).

Immunohistochemical staining for immune cells reveals that early graft infiltration is characterized by the presence of innate immune cells such as $CD11b^+$ macrophages and neutrophils beginning on 2 days post-transplantation and their numbers steadily increase to the time of graft rejection at 7 days post-transplantation (Table 2-2). Eosinophils were also detected within the graft site, however, their numbers did not appreciably increase until 4 days post-transplantation (Table 2-2). In comparison, the presence of adaptive immune cells within the NPI xenograft was delayed in the rejection process. $CD4^+$ T cells and $CD8^+$ T cells were first detected within the graft at 4 days post-transplantation whereas $CD19^+$ B cells were not detected until 5 days post-transplantation (Table 2-2). Therefore, while both innate immune cells and adaptive

immune cells were present within rejecting NPI xenografts, early infiltration by macrophage and neutrophils did not correlate with substantial β cell destruction, whereas subsequent infiltration by eosinophils, $CD4^+$ T cells, $CD8^+$ T cells and $CD19^+$ B cells were all temporally associated with substantial graft destruction.

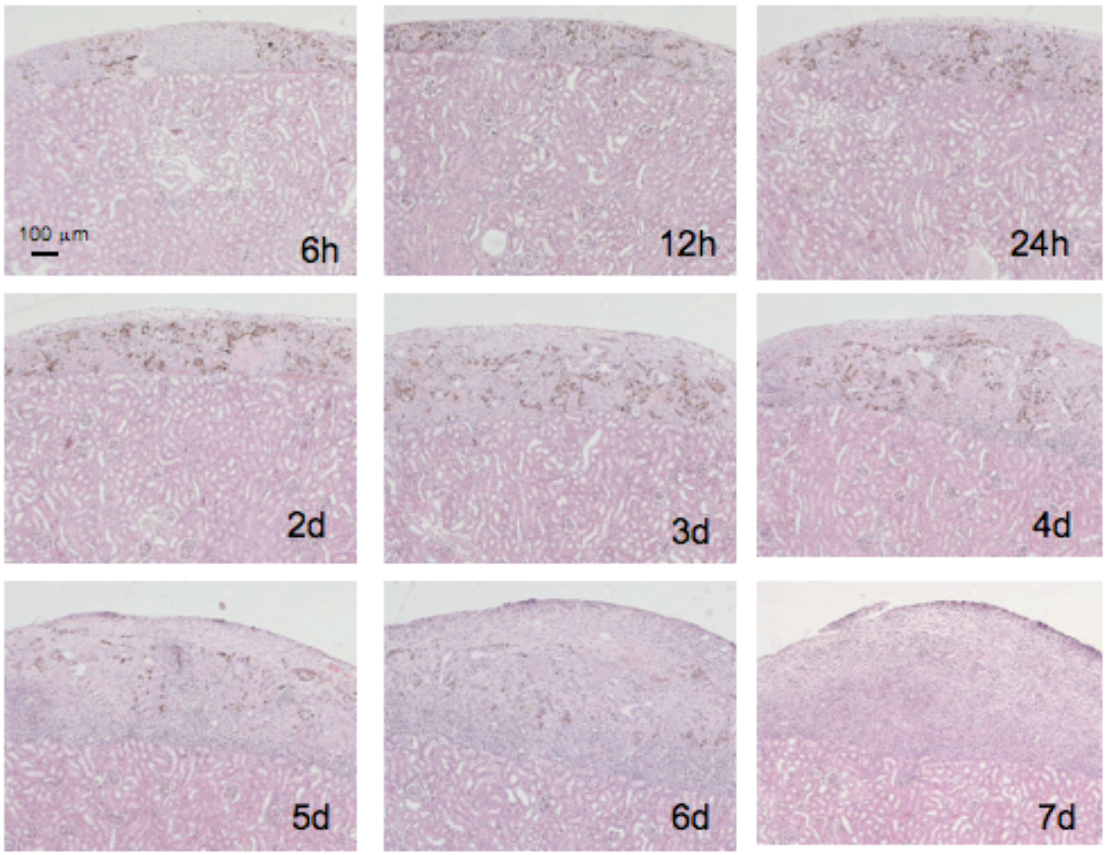

Figure 2-1: Insulin immunohistochemical staining of NPI xenografts in non-autoimmune prone, non-diabetic B6 mice at various early time-points post-transplantation. Immune cell infiltration of the NPI xenograft occurs initially on day 2 at the periphery of the graft. By day 7 post-transplantation the NPI xenograft is infiltrated completely by immune cells and no intact insulin staining islet cells are detected.

Table 2-2: Immunohistochemical staining of NPI xenografts in non-autoimmune prone, non-diabetic B6 mice. A) Rejection of NPI xenografts is characterized by initial infiltration of innate immune cells. Macrophages and neutrophils are present early at the periphery of the graft 2 days post-transplantation. Eosinophil infiltration occurs later at 4 days post-transplantation. B) Adaptive immune cell infiltration of rejecting NPI xenografts is delayed compared to innate immune cells. $CD4^+$ T cells and B cells do not infiltrate the graft site until 4 days post-transplantation. $CD8^+$ T cells are last to arrive at the graft site at 5 days post-transplantation.

Immune Cells	**Time Post-Transplantation**								
	6h	**12h**	**1d**	**2d**	**3d**	**4d**	**5d**	**6d**	**7d**
A) Innate									
$CD11b^+$ Macrophage	-	-	-	+	+	++	++	++	+++
Neutrophils	-	-	-	+	+	+	+	++	++
Eosinophils	-	-	-	-	-	+	++	++	+++
B) Adaptive									
$CD4^+$ T cells	-	-	-	-	-	+	++	++	+++
$CD19^+$ B cells	-	-	-	-	-	+	++	++	++
$CD8^+$ T cells	-	-	-	-	-	-	+	++	++

(-) no staining; (+) <20% of graft; (++) 20-50% of graft; (+++) >50% of graft

2.3.2 HUMORAL IMMUNE RESPONSE TO NEONATAL PORCINE ISLET XENOGRAFTS

Humoral immune response at early time points of NPI xenograft rejection were also examined. Mouse anti-porcine IgG antibody levels were not detected until 5 days post-transplantation and continued to increase until the time of NPI xenograft rejection at 7 days post-transplantation (Figure 2-2). These results correspond with the delayed appearance of both $CD4^+$ T cells at 4 days post-transplantation and $CD19^+$ B cells at 5 days post-transplantation (Table 2-2) indicating that B-cell activation requires $CD4^+$ T cell help.

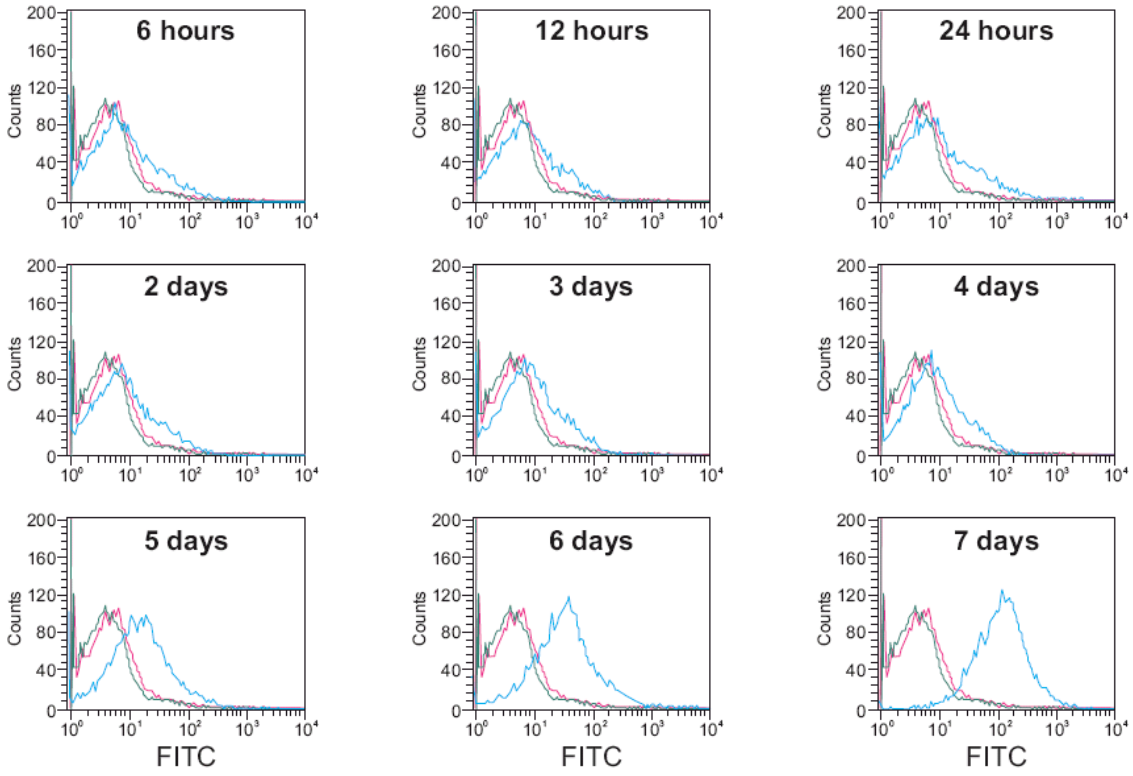

Figure 2-2: Mouse anti-porcine antibody levels in non-autoimmune prone, non-diabetic B6 mice at early time points of NPI xenograft rejection. Anti-porcine IgG antibody levels begin increasing on day 5 post-transplantation and continue to increase until time of NPI xenograft rejection on day 7.

2.3.3 NEONATAL PORCINE ISLET XENOGRAFT REJECTION IS ASSOCIATED WITH EARLY PRODUCTION OF CHEMOKINES AND TRANSIENT PRODUCTION OF CYTOKINES

Chemokine mRNA expression profiles were compared at various time points post-transplantation between rejecting NPI xenografts and control sham operated kidneys using RT PCR techniques. The expression of chemokines MCP-1, MIP-1α, MIP-1β and RANTES was detected in both transplanted islet graft sites and sham operated transplant sites (Figure 2-3). In naïve B6 mouse kidney tissue samples unaltered by surgical manipulation of the kidney capsule, the chemokines MCP-1, MIP-1α and RANTES were also detected. In contrast, expression of the chemokine MIP-1β was noticeably absent in naïve kidney samples (Figure 2-3). Therefore, this pattern of expression would suggest that expression of the chemokine MIP-1β is induced by the process of surgical manipulation of the kidney and/or injection of media within the transplant site.

In a similar fashion, locally expressed cytokine mRNA transcripts were detected in NPI transplanted and sham operated B6 mice. The cytokines IFNγ, IL-4 and IL-10 were not detected in naïve B6 mouse kidney samples nor in sham operated kidney sites (Figure 2-4). However, expression of IFNγ was seen transiently from 3 to 5 days post-transplantation corresponding to the presence of macrophage and subsequent appearance of $CD4^+$ T cells (Table 2-2). The appearance of $CD4^+$ T cells within the graft site at 4 days post-transplantation (Table 2-2) corresponded to both the transient expression of IL-4 from 4 to 6 days post-transplantation and the transient expression of IL-10 from 4 to 5 days post-transplantation (Figure 2-4). Expression of TGFβ was detected in NPI transplanted graft sites, sham operated sites throughout the rejection process as well as faintly in non-transplanted naïve B6 kidney tissue samples (Figure 2-4).

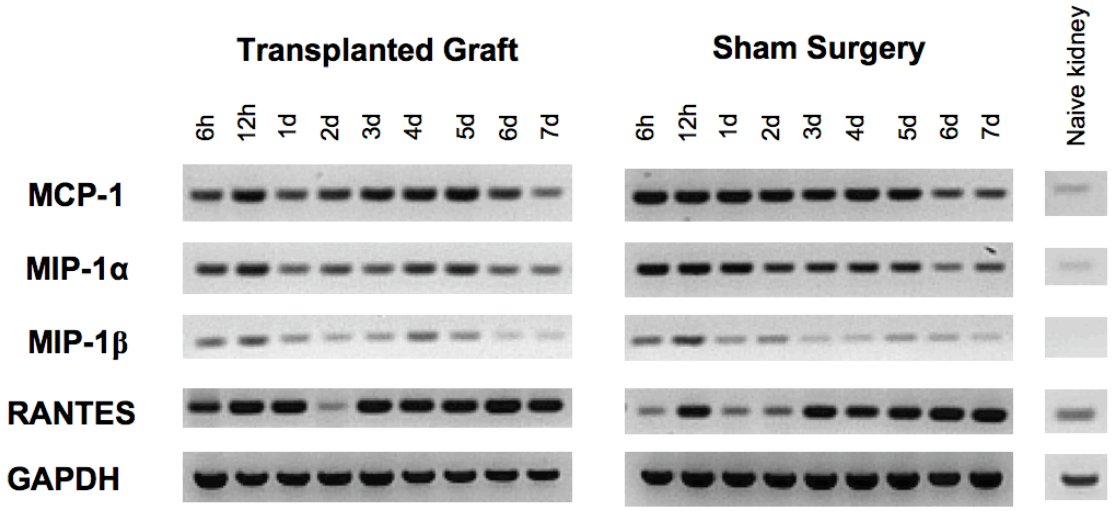

Figure 2-3: Mouse chemokine mRNA transcripts within NPI transplanted and sham transplanted graft sites in non-autoimmune prone, non-diabetic B6 mice. Transplanted grafts and surrounding kidney parenchyma were collected and cDNA was generated from mRNA transcripts using RT PCR. cDNA samples were subsequently analyzed by PCR analysis for the presence of MCP-1, MIP-1α, MIP-1β and RANTES sequences. GAPDH was used as an endogenous housekeeping gene. PCR products were separated on an agarose gel.

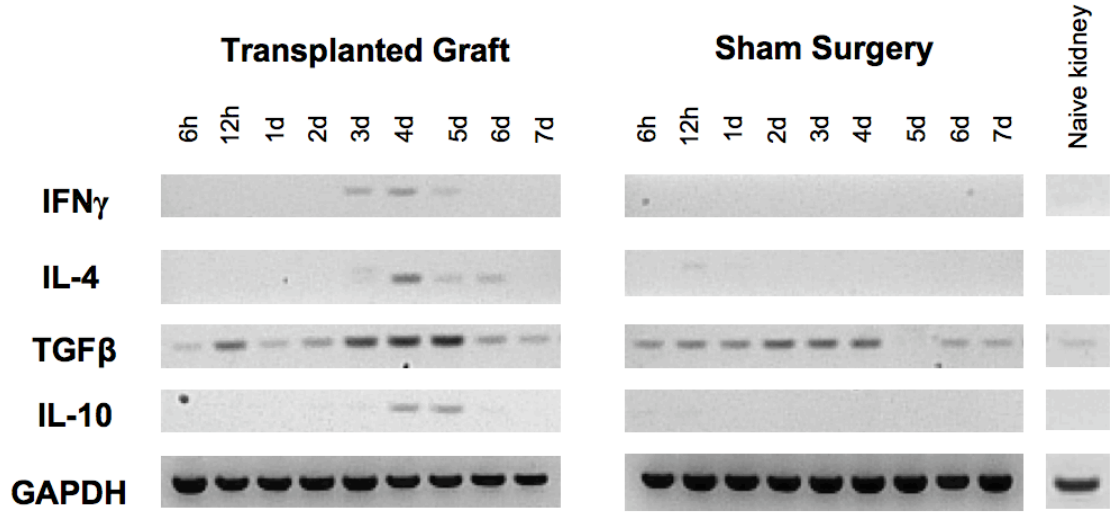

Figure 2-4: Mouse cytokine mRNA transcripts within NPI transplanted and sham transplanted graft sites in non-autoimmune prone, non-diabetic B6 mice. Transplanted grafts and surrounding kidney parenchyma were collected and cDNA was generated from mRNA transcripts using RT. cDNA samples were subsequently analyzed by PCR analysis for the presence of IFNγ, IL-4, TGFβ and IL-10 sequences. GAPDH was used as an endogenous housekeeping gene. PCR products were separated on an agarose gel.

2.3.4 NEONATAL PORCINE ISLET XENOGRAFT REJECTION DOES NOT RESULT IN ALTERATIONS IN IMMUNE CELL PROFILES NOR SYSTEMIC CHEMOKINE/CYTOKINE SIGNALS

We were also interested in determining whether systemic changes in immune cell profiles and chemokine/cytokine levels corresponded with the local graft site immune cell infiltration and chemokine/cytokine expression following NPI xenograft transplantation. Flow cytometry was used to examine $CD4^+$ T cell, $CD8^+$ T cells and $CD19^+$ B cell populations in spleen cells and $CD49b^+$ NK cells in the peripheral blood. Over the course of the NPI xenograft rejection process from 1 to 7 days post-transplantation, there was no evidence of $CD4^+$ T cell nor $CD8^+$ T cell expansion (Figure 2-5) despite the fact that both immune cell types appear and increase in numbers at the rejecting graft site at 4 days and 5 days post-transplantation (Table 2-2). The $CD19^+$ B cell population was significantly higher in sham operated B6 mice at 2 days and 5 days post-transplantation, however, overall there was no significant change in $CD19^+$ B cell populations over the course of NPI xenograft rejection in comparison to sham operated mice (Figure 2-6) despite the fact that $CD19^+$ B cells appear at the graft site beginning at 4 days post-transplantation and increase in numbers until the time of rejection (Table 2-2). Lastly, peripheral blood $CD49b^+$ NK cells were examined by flow cytometry and no significant change was seen in the systemic population of NK cells over the course of the rejection process (Figure 2-7). A limitation of this study is the lack of a monoclonal antibody specific for NK cells for the purpose of immunohistological analysis in mice. However, using flow cytometry to examine intragraft cell populations, Karlsson-Parra and colleagues have demonstrated that in the process of fetal porcine islet xenograft rejection in B6 mice 15% of infiltrating immune cells are $CD49b^+$ NK cells (5).

Therefore, despite the fact that NK cells appear to infiltrate porcine islet xenografts, their systemic population does not change over the course of rejection.

Serum chemokine and cytokine levels were measured by multiplex bead immunoassay in NPI transplanted and sham operated B6 mice at 2 days, 4 days, 5 days and 7 days post-transplantation. In NPI transplanted B6 mice, the chemokine MCP-1 did not differ significantly at all time points post-transplantation from naïve B6 mice (33.2 ± 0.0 pg/ml, n=3, Figure 2-8A). The chemokine MIP-1α was undetectable in both naïve B6 mice as well as in transplanted mice at all time points post-transplantation (n.d., n=3, Figure 2-8B). Serum IFNγ levels in transplanted B6 mice did not significantly differ from naïve B6 mice (0.7 ± 0.3 pg/ml, n=3) at all time points post-transplantation (Figure 2-8C). Similarly, IL-4 levels were not appreciably changed in NPI transplanted mice in comparison to naïve B6 mice (16.2 ± 5.5 pg/ml, n=3, Figure 2-8D). Lastly, the cytokine IL-10 was not detectable in the serum of naïve B6 mice, NPI transplanted mice nor sham operated mice at all time points (Figure 2-8E). Therefore, it appears that while intragraft cytokines IFNγ, IL-4 and IL-10 are transiently upregulated in NPI transplanted mice (Figure 2-4), this does not result in a systemic production of these same cytokines.

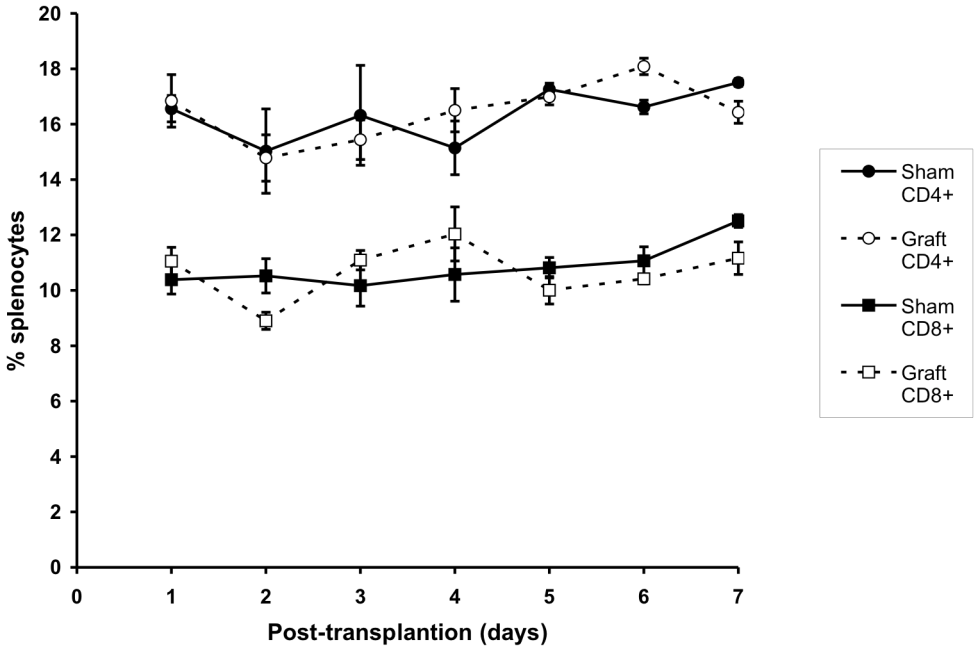

Figure 2-5: $CD4^+$ and $CD8^+$ T cell populations in the spleen of non-autoimmune prone, non-diabetic B6 mice. Spleen cells from NPI transplanted and sham transplanted mouse recipients were harvested at various time points and $CD4^+$ and $CD8^+$ T cell populations were examined by flow cytometry. Both systemic $CD4^+$ and $CD8^+$ T cells were unaltered during the course of NPI xenograft rejection.

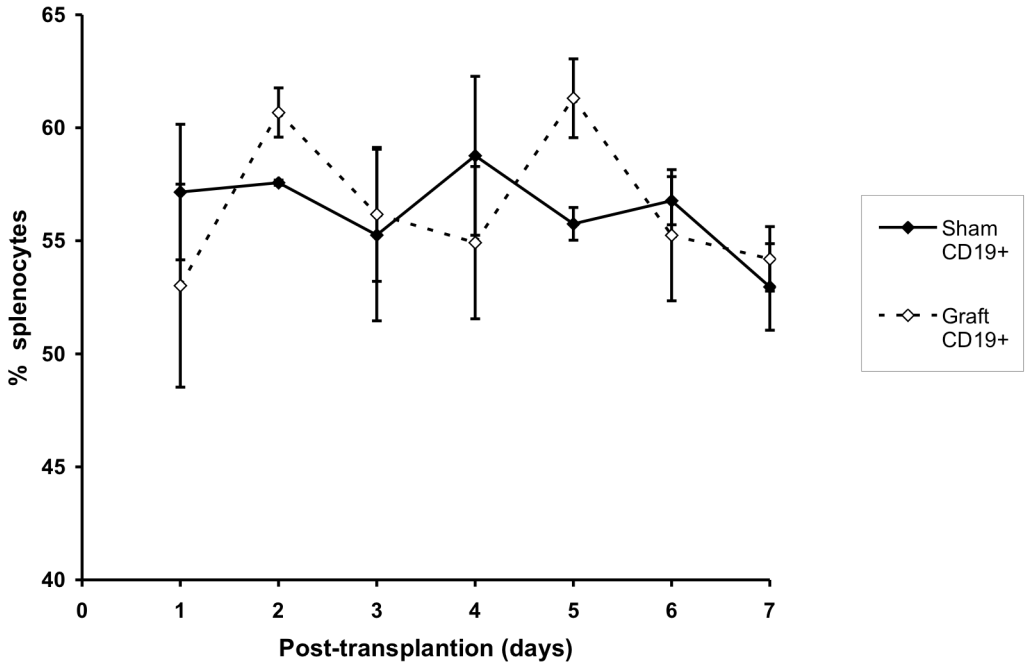

Figure 2-6: CD19$^+$ B cell population in the spleen of NPI transplanted and sham transplanted non-autoimmune prone, non-diabetic B6 mice. Spleen cells from NPI transplanted and sham transplanted mouse recipients were harvested at various time points and the CD19$^+$ B cell population was examined by flow cytometry. Systemic CD19$^+$ B cells were unaltered during the course of NPI xenograft rejection.

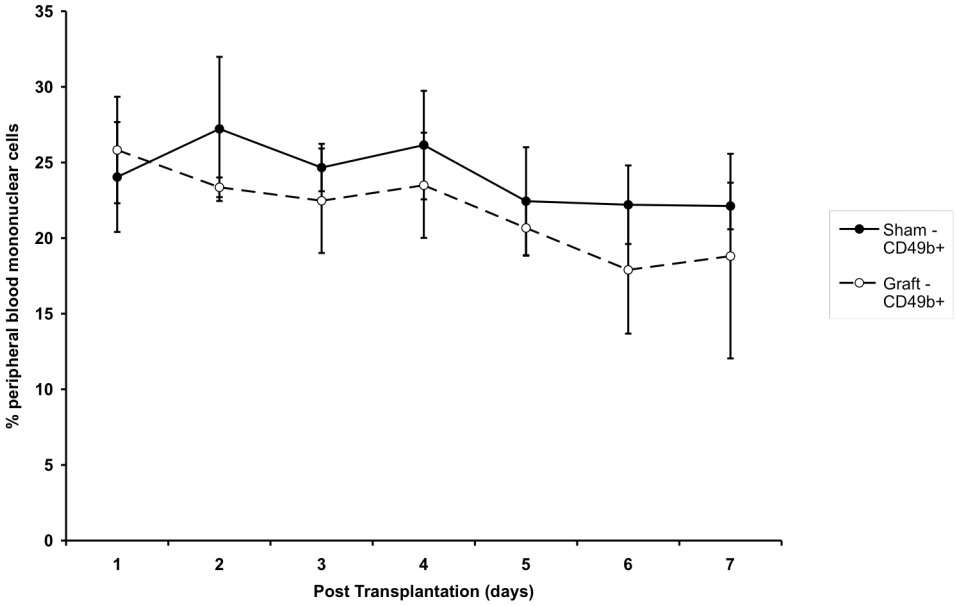

Figure 2-7: CD49b^{+} NK cell population in the peripheral blood of NPI transplanted and sham transplanted non-autoimmune prone, non-diabetic B6 mice. Peripheral blood from NPI transplanted and sham transplanted mouse recipients was harvested at various time points and the CD49b^{+} NK cell population was examined by flow cytometry. Systemic CD49b^{+} NK cells were unaltered during the course of NPI xenograft rejection.

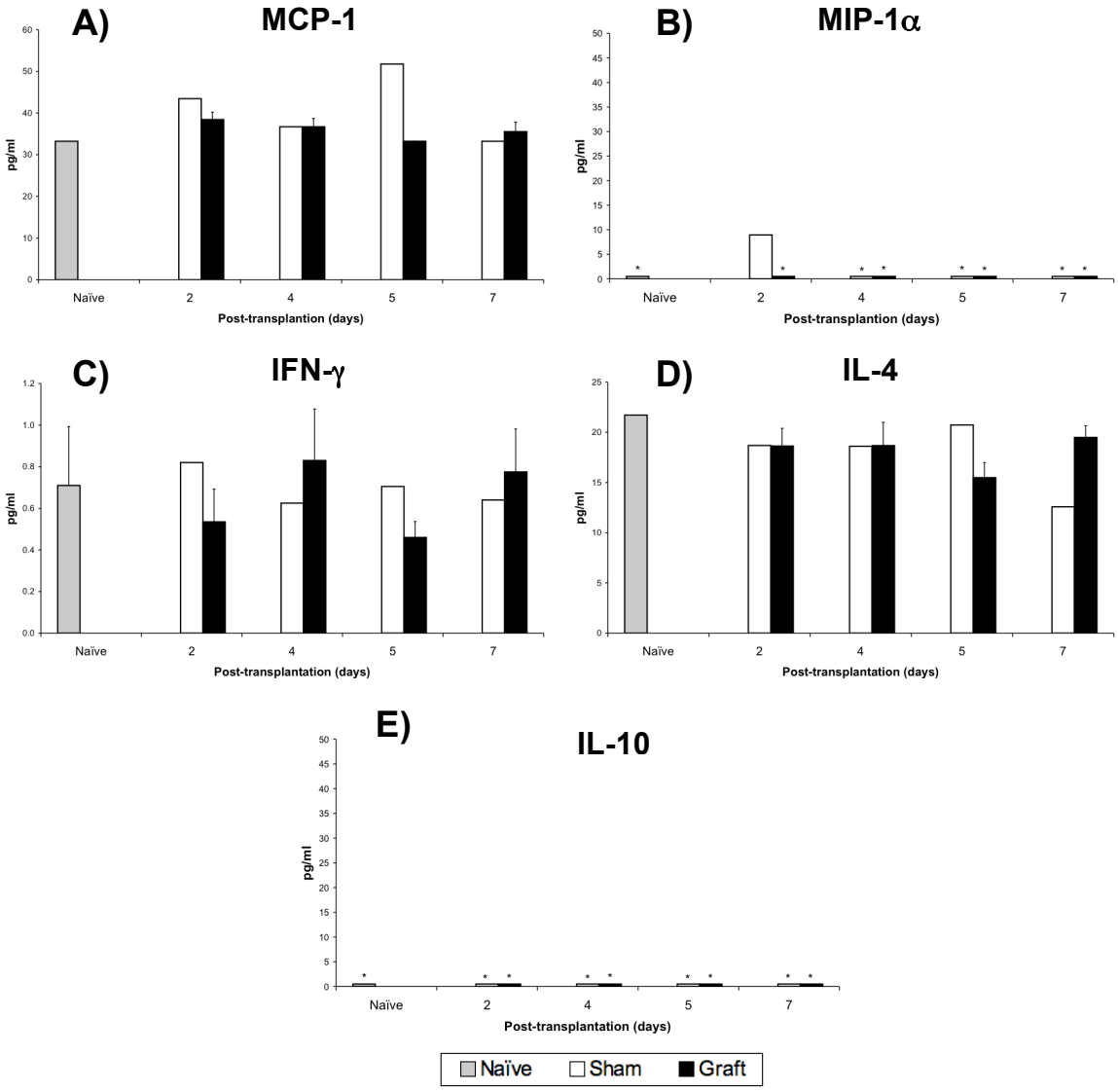

Figure 2-8: Serum chemokine levels in NPI transplanted and sham transplanted non-autoimmune prone, non-diabetic B6 mice. A) MCP-1, B) MIP-1α, C) IFNγ, D) IL-4, E) IL-10. (* chemokine/cytokine levels not detected)

2.4 DISCUSSION

We have previously demonstrated that the rejection process of NPI xenografts is critically dependent on the presence of $CD4^+$ T cells (4). In this study, we examined the early immunologic events leading to the rejection of NPI xenografts in an attempt to further elucidate the mechanism of NPI xenograft rejection in the absence of islet β cell specific autoimmunity. We have demonstrated that expression of the chemokine MIP-1β at the graft site occurs but does not appear to be specific to the process of NPI xenograft rejection but rather surgical manipulation of the graft site (Figure 2-3). Other chemokines such as MCP-1, MIP-1α, and RANTES were also detected at the graft site, however, these chemokines were also present endogenously in naïve B6 kidney parenchyma (Figure 2-3). These results compare favorably to those of Solomon and colleagues who found that transplantation of fetal porcine islets under the kidney capsule in a mouse model also produced MCP-1, MIP-1α, MIP-1β and RANTES in the early post-transplantation period (6-8). Furthermore, the ability for chemokines to act as chemoattractant may be preserved across species and chemokines secreted by porcine islets have the ability to induce migration of monocytes (9). One limitation of our study was the use of non-quantitative PCR techniques to examine the presence of these chemokines.

While MCP-1, MIP-1α and RANTES were detected faintly within naïve B6 mouse kidney, it is possible that quantitative real-time PCR techniques could detect whether these chemokines are found in greater quantities following surgical manipulation of the graft site, similar to MIP-1β. The chemokines MCP-1, MIP-1α, MIP-1β and RANTES are all associated with the migration of macrophages, dendritic cells and

activated T cells (10, 11) while MIP-1α, MIP-1β and RANTES are additionally associated with migration of natural killer cells (10). The expression of chemokines locally within the graft site (Figure 2-3) but not systemically in recipient serum (Figure 2-8) is consistent with their role in trafficking leukocytes by means of chemotactic concentration gradient to sites of immune activity. Furthermore, the presence of these chemokines within the graft site may be responsible for the migration of macrophage and T cells observed within the graft site (Table 2-2). Transplantation technique certainly influences the degree of tissue damage and inflammation at the graft site. This current study and that by Solomon and colleagues (6) utilized the widely accepted experimental model of islet transplantation under the kidney capsule. However currently, clinical islet transplantation is performed via portal vein infusion. Hardstedt and colleagues examined the chemokine profile within the hepatic parenchyma of rhesus macaques following intraportal transplantation of adult porcine islets. When transplanted intraportally, a slightly different chemokine profile was observed with significant increases in both MIP-1α and RANTES transcripts, but no significant change in MIP-1β nor MCP-1 transcripts between early and late time points (12). Differences in chemokine expression profiles in porcine islets transplanted under the kidney capsule compared to those transplanted intraportally may potentially be a result of local tissue damage after transplantation and/or degree of local ischemia.

Targeting chemokines in the prevention of NPI xenograft rejection certainly is an attractive option which could prevent immune cell migration to the graft site without significantly altering normal immune cell function. Potential strategies to target the function of chemokines in recruiting immune cells to the transplant graft site are to

administer modified chemokines which may act as antagonists (13-15), administer small non-peptide molecules which similarly act as antagonists to chemokine receptors (16, 17) or by administration of blocking monoclonal antibodies targeted to chemokine receptors (18). Currently, the use of specific chemokine antagonists have not been investigated in the prevention of porcine islet xenograft rejection. However, this approach has been studied in the treatment of asthma and has been demonstrated to ameliorate associated airway inflammation (19). Any therapies incorporating the use of chemokine antagonism, however, will likely require targeting of multiple chemokines. Solomon and colleagues demonstrated that while transplantation of fetal porcine islets to CCR2 deficient mice (lacking major receptor for MCP-1) and CCR5 deficient mice (lacking the co-receptor for MIP-1α, MIP-1β and RANTES), did delay intragraft recruitment of macrophages but $CD4^+$ T cells graft rejection was not prevented (6).

In this study we have demonstrated that NPI xenograft rejection is associated with initial innate immune cell infiltration of the graft and subsequently followed by infiltration of the graft at 4 days post-transplantation by $CD4^+$ T cells (Table 2-2). It is currently unclear whether early innate immune cell infiltration plays a vital role in NPI xenograft rejection. Specifically, recipient macrophage play an important role as antigen presenting cells (APCs) in discordant islet xenograft models (20). Depletion of macrophage appears to delay porcine islet xenograft rejection (20, 21), however, xenografts ultimately undergo rejection suggesting secondary pathways of antigen presentation exists. Therefore, early infiltration of macrophage is likely to play a role in initiating islet xenograft rejection, and while preventing their accumulation at the graft site may help prevent rejection this strategy is unlikely to be sufficient in of itself.

While $CD4^+$ T cells increase in number locally at the graft site, there does not appear to be a corresponding expansion of the $CD4^+$ T cell population systemically (Figure 2-5). Corresponding with the appearance of $CD4^+$ T cells at the graft site is local production of cytokines IFNγ, IL-4 and IL-10 (Figure 2-4). IFNγ is produced by $CD4^+$ Th1 cells and activates macrophages, while IL-4 and IL-10 are produced by Th2 cells. The production of IFNγ is important in both macrophage and NK cell activation. Yi and colleagues have demonstrated that inhibition of IFNγ by monoclonal antibody administration can delay porcine islet xenograft rejection by preventing the activation of both macrophages and NK cells (22). The production of the Th2 cytokine IL-4 on days 4 to 6 post-transplantation corresponds with the production of anti-porcine antibody observed beginning on day 5 post-transplantation (Figure 2-2). The role of IL-10 in NPI xenograft rejection currently is not clear. One possibility is that IL-10 regulates the response of activated $CD4^+$ T cells (23) which would correspond to the transient production of IL-10 on days 4 and 5 days post-transplantation, the time at which $CD4^+$ T cells are recruited to the graft site and produce IFNγ and IL-4.

Due to the observational nature of our study it is difficult to link causality between the different immune events that have occurred. While the presence of eosinophils was demonstrated at the graft site during the rejection process, congruent with previous studies (6, 22, 24, 25), it has been previously demonstrated that they are possibly innocent bystanders as they do not play an important role in porcine islet xenograft rejection (22, 24). Therefore, the results presented in this study serve to identify other potential targets for immune therapy in the prevention of NPI xenograft

rejection. We postulate that simultaneous targeting of these targets holds more promise in promoting long-term survival of NPI xenografts.

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CHAPTER THREE

DIFFERENTIAL ROLE OF NATURAL KILLER CELLS IN RAT VERSUS NEONATAL PORCINE ISLET XENOGRAFT REJECTION IN IMMUNE-COMPETENT MICE

3.1 INTRODUCTION

The field of islet transplantation has seen significant advances since the first successful islet transplantation by Lacy over 35 years ago (1). While clinical islet transplantation has proven to be an effective treatment for brittle type 1 diabetic patients (2), a limiting factor remains the scarcity of human donor islets for transplantation. Islet xenotransplantation using porcine islets is a potentially viable solution to the shortage of islet donor supply; however, preventing cell-mediated rejection remains an ongoing obstacle (3, 4).

Natural killer (NK) cells may play a role in islet xenograft rejection given their temporal and spatial association with rejecting adult porcine islet xenografts (5, 6). NK cells are lymphoid cells derived from bone marrow that share a common early progenitor to T cells. Like T cells, NK cells can act as cytotoxic effector cells by perforin and granzyme degranulation. A major difference, however is that NK cells do not express antigen specific receptors, rather, a collection of receptors which provide activation and inhibitory signals control the activity of NK cells (7). Engagement of self-MHC I receptors is a major mechanism by which NK-mediated damage to self tissues is avoided (8, 9). NK cells function as potent effector cells of rejection of allogeneic bone marrow transplants (10), vascularized solid organ transplants (11, 12) and play a role in promoting transplant tolerance (13). Currently, however, it is unclear what role NK cells play in neonatal porcine islet (NPI) xenograft rejection.

There is mounting evidence that the mechanism of islet xenograft rejection depends on the phylogenetic disparity between host and donor islet species (14, 15). While rejection of rat islet xenografts in mice are not dependent on the presence of

macrophage (14), human and porcine islet xenograft rejection in mice are dependent on the presence of macrophage (14, 16). Furthermore, while both rat and porcine islet xenograft rejection are $CD4^+$ T cell mediated processes, there is a marked hierarchy in the requirement for host MHC class II-restricted indirect antigen presentation with porcine islet xenograft rejection completely dependent on this pathway (15). Therefore, it appears that the mechanism by which islet xenografts are rejected may differ depending on the phylogenetic disparity between the host and donor islet species.

The aim of the present study was to compare the role of NK cells in concordant and discordant islet xenograft rejection. We examined *in vivo* the effect of depleting NK cells using anti-NK1.1 monoclonal antibody (mAb) on the rejection of concordant rat islet and discordant NPI xenografts transplanted in mice.

3.2 MATERIALS AND METHODS

3.2.1 ANIMALS

Male Wistar-Furth rats (200-300 grams, Harlan, Indianapolis, IN, USA) and 1-3 day old Landrace-Yorkshire neonatal pigs of either sex (1.5-2 kg, University of Alberta farm, Edmonton, AB, Canada) were used as islet donors. Six to 8 weeks old male C57BL/6J (B6, H-2^b, Jackson Laboratory, Bar Harbor, ME, USA) and immune-deficient C57Bl/6-rag1 $^{tm1/mom}$ (B6 *rag*-/-, H-2^b, Jackson Laboratory) mice were used as recipients. All animals were fed standard laboratory food and cared for according to the guidelines established by the Health Sciences Animal Policy and Welfare Committee at the University of Alberta.

3.2.2 RAT ISLET ISOLATION

Islet isolation and transplantation was performed as previously described (17). Briefly, Wistar-Furth rats were anesthetized and pancreatic ducts were distended *via* bile duct cannulation with HBSS (Sigma, St Louis, MO, USA) prior to excision. Pancreases were cut into small pieces then digested with 3.75 mg/ml collagenase (Sigma). Islets and exocrine tissue were isolated following Dextran (Sigma) density gradient separation. Islets were hand picked, counted and cultured overnight at 37˚C, 5% CO_2, 95% air in 10 ml of Ham's F10 media (Gibco, Burlington, ON, Canada).

3.2.3 NEONATAL PORCINE ISLET ISOLATION

Islets from neonatal pigs were isolated as previously described (18). Briefly, neonatal pigs were anesthetized and subjected to laparotomy and exsanguinated. The pancreas was removed, placed in Hank's Buffer Salt Solution (HBSS, Sigma), cut into small pieces and digested with 2.5 mg/ml collagenase (Sigma). Digested tissue was filtered through a 500 μm nylon screen then cultured for 7 days in Ham's F10 medium containing 10 mmol/l glucose, 50 μmol/l isobutylmethylxanthine (ICN Biomedicals, Montreal, PQ, Canada), 0.5% bovine serum albumin (fraction V, radioimmunoassay grade; Sigma), 2 mmol/l L-glutamine, 3 mmol/l $CaCl_2$, 10 mmol/l nicotinamide (BDH Biochemical, Poole, England), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C, 5% CO_2, 95% air. A complete media change was performed on culture days 1, 2, 4 and 6.

3.2.4 EXPERIMENTAL DESIGN

Recipient mice were rendered diabetic by intraperitoneal (i.p.) injection of 180 mg/kg streptozotocin (Sigma) for B6 mice and 175 mg/kg for B6 *rag-/-* mice 4-6 days before transplantation. Mice with two consecutive non-fasting blood glucose levels of ≥17 mmol/l were used as recipients. Five hundred Wistar-Furth rat islets or 2000 NPI were transplanted under the left kidney capsule of diabetic B6 and B6 *rag-/-* mice. Recipient B6 mice received no treatment or continuous i.p. administration of anti-NK1.1 mAb at a dose of 100 μg on day –1 pre-transplant, 25 μg on days 0-14 inclusively post-transplant, and 25 μg once per week thereafter (PK136, mouse IgG2a, Bio Express Inc., West Lebanon, NH, USA). B6 *rag-/-* mouse recipients received no treatment. Islet graft function was monitored by blood glucose measurements two times a week. Graft function was defined as blood glucose values ≤8.4 mmol/l while graft rejection was defined as the first of three consecutive days of blood glucose values >10 mmol/l and was further confirmed by histological analysis of the islet graft. Functionality of islet grafts was confirmed by the ability to reverse diabetes in immune-deficient B6 *rag-/-* mouse recipients. Furthermore, in mouse recipients with long-term (>100 days) graft survival, return to hyperglycemia following nephrectomy of the graft-bearing kidney established that normoglycemia was graft-dependent.

3.2.5 IMMUNOHISTOLOGICAL ANALYSIS

Graft-bearing kidneys were harvested following rejection or >100 days post-transplantation and fixed in 10% buffered formalin solution and embedded in paraffin. Insulin staining was performed on 5 μm sections of the graft. Graft sections on slides

were incubated for 30 min with guinea pig anti-insulin antibody (1:1000 dilution, Dako Laboratories, Mississauga, ON, Canada) followed by a 20 min incubation with biotinylated goat anti-guinea pig IgG secondary antibody (1:200 dilution, Vector Laboratories, Burlington, CA, USA). Sections were then incubated with avidin-biotin complex/horseradish peroxidase for 40 min (Vector Laboratories) using 3, 3-diaminobenzidinetetrahydrochloride (BioGenex, San Ramon, CA, USA) as substrate. A positive reaction was detected within 5 min. Graft sections on slides were counterstained with Harris' hematoxylin and eosin. Negative control staining for this experiment was performed by omitting the primary antibody.

3.2.6 DETECTION OF NATURAL KILLER CELLS IN THE PERIPHERAL BLOOD

Peripheral blood from untreated naïve B6 mice and anti-NK1.1 mAb treated mice was collected by cardiac puncture and anti-coagulated by mixing with 500 i.u. of heparin (LEO Pharma Inc., Thornhill, ON, Canada). Mononuclear cells were isolated by lympholyte mammal (Cedarlane, Burlington, ON, Canada) gradient separation. Subsequently, 5 x 10^5 peripheral blood mononuclear cells (PBMCs) were incubated for 30 min at 4˚C with FITC-conjugated anti-CD49b/Pan-NK cell mAb diluted 1:100 (DX5, BD Biosciences Pharmingen, Mississauga, ON, Canada) then washed two times with 1x PBS. Data were acquired using a BD FACS Calibur flow cytometer (BD Biosciences, Mississauga, ON, Canada). Viable lymphocytes were gated and the proportion of CD49b expressing cells was determined.

3.2.7 DETECTION OF MOUSE ANTI-RAT AND ANTI-PORCINE ANTIBODIES

Peripheral blood samples were collected from naïve non-transplanted and islet transplanted B6 mouse recipients. Blood samples were allowed to clot for 30 min then spun at 10,000 rpm for 10 min to isolate the serum. Spleen cells (1×10^6) from donor Wistar-Furth rats and donor Landrace-Yorkshire neonatal pigs were incubated in 1 ml of a 1:128 dilution of mouse serum for 1 hour at 37˚C, 5% CO_2, 95% air. Spleen cells were then washed with 1x PBS and incubated with a 1:200 dilution of FITC-conjugated rat adsorbed goat anti-mouse IgG mAb (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) for 1 hour at 4˚C. Spleen cells were then washed two times in 1x PBS and suspended in 500 μl of FACS buffer (1x PBS, 2% FBS). The percentage of rat and NPI spleen cells bound to mouse antibody was determined from fluorescence histograms on a BD FACS Calibur flow cytometer (BD Biosciences) after gating on viable lymphocytes. Controls for this experiment included donor spleen cells incubated without mouse serum or with mouse serum but no secondary antibody added.

3.2.8 DETECTION OF T CELL POPULATIONS IN MOUSE SPLEEN CELLS

One million spleen cells from naïve non-transplanted or islet transplanted B6 mouse recipients were incubated for 30 min at 4˚C with 1:100 dilution of PE-conjugated anti-CD4 and anti-CD8 mAbs (BD Biosciences Pharmingen), then washed 2 times with 1x PBS. The percentage of $CD4^+$ and $CD8^+$ T cells were determined from fluorescence histograms created using a BD FACS Calibur flow cytometer (BD Biosciences). Controls for this experiment included spleen cells that were not incubated with antibodies.

3.2.9 STATISTICAL ANALYSIS

Statistical differences in graft survival between groups and the percentage of NK cells found in PBMCs were determined by Kaplan-Meier log rank test and a Student's t-test, respectively, using SPSS statistical software, version 11.5 for Windows (SPSS Inc., Chicago, IL, USA). A p-value <0.05 was considered to be statistically significant for both statistical tests.

3.3 RESULTS

3.3.1 ANTI-NK1.1 MONOCLONAL ANTIBODY ADMINISTRATION RESULTS IN LONG-TERM SURVIVAL OF RAT BUT NOT PORCINE ISLET XENOGRAFTS

We first determined the effect of anti-NK1.1 mAb administration in B6 mouse recipients of rat islets and NPI. B6 mouse recipients of rat islets treated continuously with anti-NK1.1 mAb had a significantly ($p=0.001$) prolonged mean-graft survival (>84.7 days, n=7) as compared to untreated controls (14.4 days, n=9) with five of seven mice attaining long-term graft function (>100 days; Table 3-1). The function of the grafts was correlated to the histological morphology of rat islet xenografts either at the time of rejection or at >100 days post-transplantation for those mice attaining long-term graft function. Rejected rat islet grafts from untreated B6 mouse recipients were devoid of insulin positive cells (Figure 3-1A). While, rat islet grafts from, anti-NK1.1 mAb treated mice demonstrated the presence of insulin positive cells and a paucity of infiltrating immune cells at >100 days post-transplantation (Figure 3-1B).

In comparison, B6 mouse recipients of NPI that were treated continuously with anti-NK1.1 mAb did not result in long-term islet xenograft survival. None of the

untreated mouse recipients attained long-term graft survival, while only one of seven mice treated continuously with anti-NK1.1 mAb achieved long-term graft function (Table 3-1). Examination of the NPI xenografts histologically at >100 days post-transplantation shows NPI xenografts from untreated mice lacked the presence of insulin positive cells (Figure 3-1C). In the lone mouse transplanted with NPI and treated with anti-NK1.1 mAb that attained long-term graft function, scant insulin positive cells were detected, despite the fact that the majority of the graft had been rejected (Figure 3-1E). Interestingly, even in mice treated with anti-NK1.1 mAb that did not attain graft function, while no insulin positive cells were detected, a dense cellular infiltrate at the NPI xenograft site was seen at > 100 days post-transplantation suggesting a delay in the rejection process (Figure 3-1D).

As NPI are an immature islet source and demonstrate delayed function, we confirmed that mice transplanted with NPI and no graft function was due to rejection of the NPI xenograft as opposed to primary non-function of the graft by transplanting NPI into immunodeficient B6 *rag-/-* mouse recipients in a parallel study. NPI demonstrated delayed maturity and rendered B6 *rag-/-* mice normoglycemic by 70.1 ± 9.5 days post-transplantation (n=17) and maintained normoglycemia for >100 days. Histological examination of NPI grafts from B6 *rag-/-* mice displayed an abundance of insulin positive cells devoid of immune cell infiltrate (Figure 3-1F). Therefore, NPI function in immune deficient B6 *rag-/-* controls confirmed function of our islets and indicate that NPI transplanted in B6 mouse recipients that remained diabetic were indeed rejected.

Table 3-1: Anti-NK1.1 mAb therapy prolongs rat islet but not neonatal porcine islet xenograft survival. Streptozotocin-induced diabetic B6 mice were transplanted with 500 rat islets or 2000 NPIs. Control B6 mouse recipients were untreated (groups 1 and 3) whereas experimental mice (groups 2 and 4) received intra-peritoneal injections of anti-NK1.1 mAb of 100 μg on day -1 pre-transplant, 25 μg from days 0-14 inclusively post-transplant and 25 μg once a week thereafter. Long-term euglycemia refers to graft function for >100 days post-transplantation.

Treatment	Islets	n	Graft Survival (days)	Mean Graft Survival (days)	Long-term Euglycemia
1) Untreated	Rat	9	8, 11, 12 (x4), 14, 24, 25	14.4 ± 2.0	0/9
2) Anti-NK1.1	Rat	7	39, 54, >100 (x5)	87. 4 ± 10.0 *	5/7 †
3) Untreated	NPI	9	0 (x9)	n/a ‡	0/9
4) Anti-NK1.1	NPI	7	0 (x6), >100	n/a ‡	1/7

* p-value: <0.05 vs. untreated rat islet xenograft
† p-value: <0.05 vs. untreated rat islet xenograft
‡ Rejected NPI xenografts did not exhibit graft function due to the delayed function of these islets. The ability of NPI xenografts to reverse diabetes was verified in immune-deficient B6 *rag-/-* mouse recipients which exhibited delayed graft function at 70.1 ± 9.5 days post-transplantation.

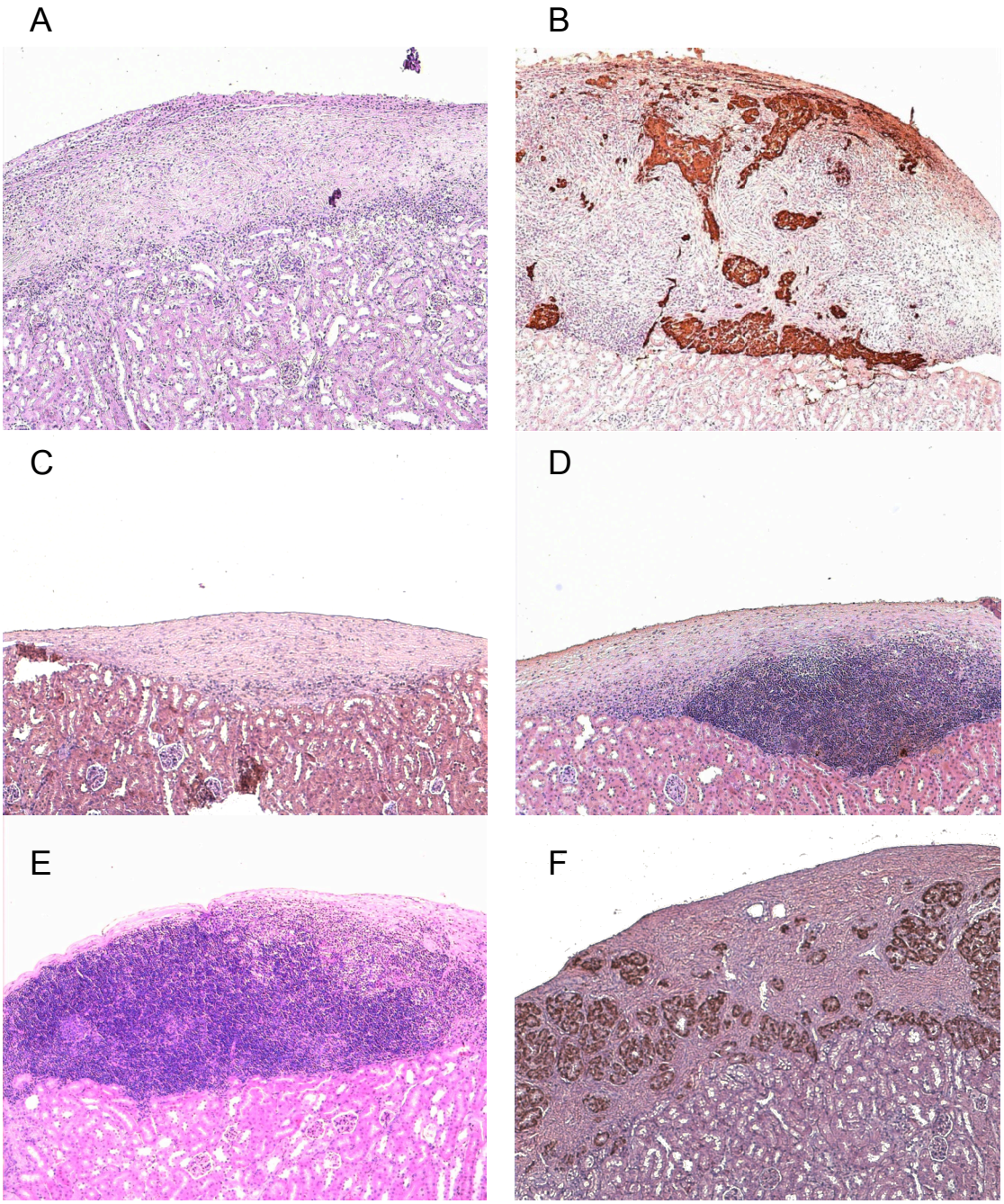

Figure 3-1: Representative gross morphology of islet xenografts in anti-NK1.1 mAb treated B6 mice. Streptozotocin-induced diabetic B6 mice were transplanted with rat islets (A, B) and NPI (C, D) under the kidney capsule. Histological examination of rat islet xenografts at time of rejection or >100 days post-transplantation in mice exhibiting long-term graft function showed an absence of insulin positive cells in untreated mice (A) but an abundance of insulin positive cells with a paucity of infiltrating immune cells in anti-NK1.1 mAb treated mice (B). NPI xenografts at >100 days post-transplantation showed an absence of insulin positive cells in both untreated (C) and anti-NK1.1 mAb treated (D) mice. Scant insulin positive cells are detected in NPI xenograft attaining function beyond 100 days post-transplantation (E). B6 *rag*-/- mice transplanted with NPI (F) demonstrate the presence of abundant insulin positive cells at >100 days post-transplantation.

3.3.2 PREVENTION OF RAT ISLET XENOGRAFT REJECTION IS ASSOCIATED WITH NATURAL KILLER CELL DEPLETION

The peripheral blood of naïve non-transplanted and islet-transplanted B6 mice that were untreated or treated with anti-NK1.1 mAb were used to determine the NK cell populations. In untreated mice that rejected the rat islet xenograft, a significant increase in the peripheral blood NK cell population (56.4 ± 3.5%, n=4) was observed as compared to naïve non-transplanted B6 mice (27.2 ± 3.5%, n=3; $p<0.01$; Figure 3-2). In contrast, there was a significant decrease in the peripheral blood NK cell population (9.0 ± 4.4%, n=3) in recipients of rat islets treated with anti-NK1.1 mAb that had long-term graft function, compared to both naïve non-transplanted ($p=0.03$) and untreated B6 mice transplanted with rat islets ($p<0.01$; Figure 3-2) suggesting that long-term graft function is associated with depletion of NK cells.

A similar trend was observed in B6 mouse recipients of NPI xenografts. Rejection of islet xenografts in untreated B6 mice was also associated with an expansion of the peripheral blood NK cell population (41.9 ± 2.5%, n=5) as compared to naïve non-transplanted B6 mice (27.2 ± 3.5%, n=3; $p=0.03$; Figure 3-3). While B6 mouse recipients of NPI xenografts treated with anti-NK1.1 mAb that rejected their graft had a significantly decreased number of peripheral blood NK cells (22.1 ± 5.2%, n=6) as compared to untreated B6 mouse recipients ($p<0.01$), this treatment did not deplete NK cells to a level significantly lower than that found in naïve non-transplanted B6 mice ($p>0.05$; Figure 3-3). The lone mouse transplanted with NPI that did achieve long-term graft function after anti-NK1.1 mAb treatment showed a similar level of peripheral blood NK cells (16.3%).

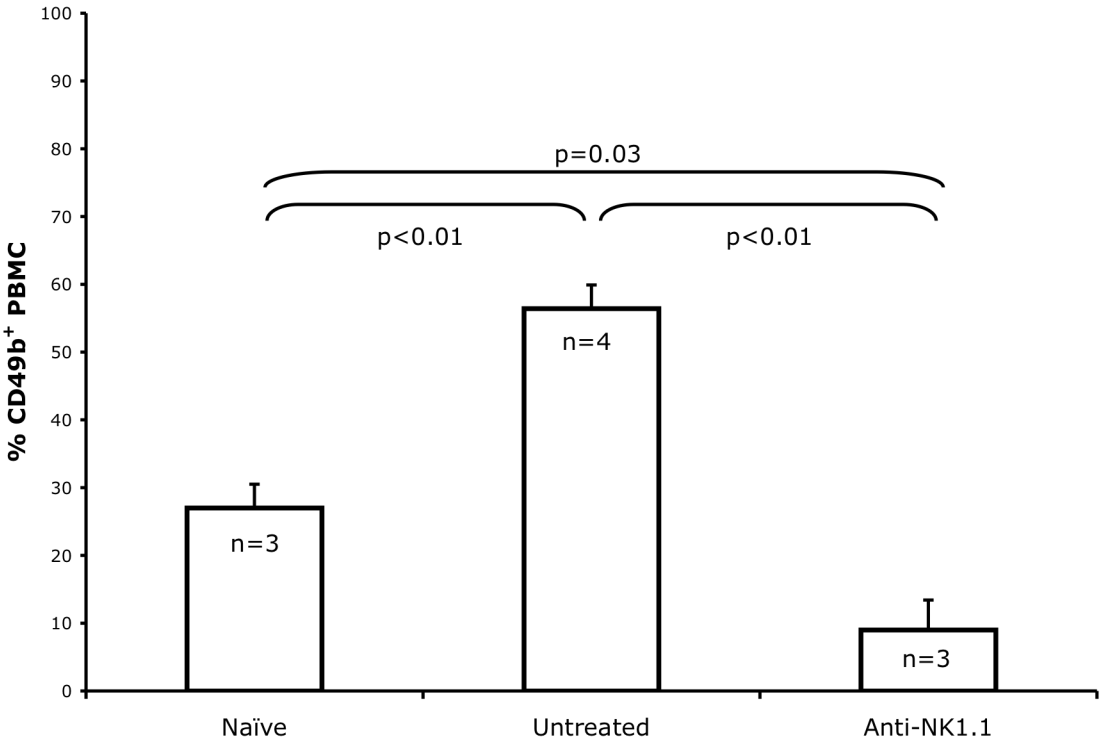

Figure 3-2: NK cell population in peripheral blood of naïve untransplanted B6 mice, untreated rat islet transplanted and anti-NK1.1 mAb treated rat islet transplanted B6 mice. Peripheral blood $CD49b^+$ cell populations were determined using flow cytometry. In comparison to naïve B6 mice, the NK cell population was markedly higher in untreated B6 mice with rejected rat islet xenografts (27.2 ± 3.5 % vs. 56.4 ± 3.5 %; $p<0.01$). In contrast, anti-NK1.1 mAb treated mice with functioning rat islet xenografts had significantly fewer NK cells (9.0 ± 4.4 %) in comparison to both naïve ($p=0.03$) and untreated B6 mice ($p<0.01$).

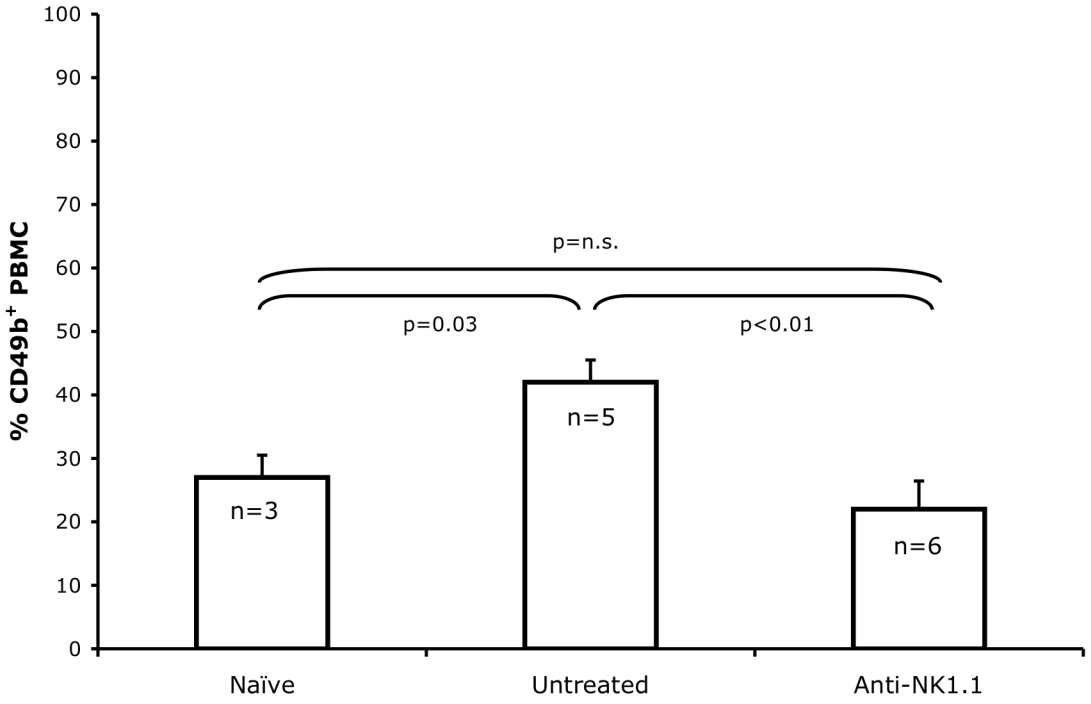

Figure 3-3: NK cell population in peripheral blood of naïve untransplanted B6 mice, untreated NPI transplanted and anti-NK1.1 NPI transplanted B6 mice. Peripheral blood CD49b$^+$ cell populations were determined using flow cytometry. Untreated B6 mice with rejected NPI xenografts had a significant increase in NK cells over naïve B6 mice (41.9 ± 2.5% vs. 27.2 ± 3.5%; p=0.03). In comparison, anti-NK1.1 mAb treated B6 mice also with rejected NPI xenografts had significantly fewer NK cells than untreated B6 mice (22.1 ± 5.2% vs. 41.9 ± 2.5%; p<0.01) but not significantly different from naïve B6 mice (22.1± 5.2% vs, 27.2 ± 3.5%; p>0.05).

3.3.3 ANTI-DONOR ANTIBODY PRODUCTION IS NOT INHIBITED BY ANTI-NK1.1 MONOCLONAL ANTIBODY THERAPY

The presence of anti-donor antibodies was detected to determine whether protection conferred by anti-NK1.1 mAb inhibited the humoral response in mouse recipients. Serum from recipient mice was incubated with donor-specific spleen cells and bound mouse anti-donor IgG antibody was detected by a flurochrome conjugated secondary antibody using flow cytometry. We found no detectable mouse anti-rat IgG

antibody present in naïve B6 mouse serum (Figure 3-4A). Anti-rat IgG Ab was detected in the serum of rejected, untreated B6 mice transplanted with rat islets with a median of 98.1% (range 98.0-98.2%, n=2, Figure 3-4B) of spleen cells bound to the IgG Ab. Anti-NK1.1 mAb treatment did not affect the anti-rat IgG Ab production as B6 mouse recipients with long-term graft survival (median 68.0%, range 10.7-100.0%, n=3, Figure 3-4C) and those that rejected their rat islet xenograft (100%, n=1) had evidence of mouse anti-rat IgG Ab production. Therefore, despite the fact that rat islet xenograft rejection is prevented by anti-NK1.1 mAb treatment, mouse anti-rat IgG Ab response is not affected by this therapy.

Similarly, in mice transplanted with NPI, mouse anti-porcine IgG Ab production was not affected by anti-NK1.1 mAb treatment. As expected, no anti-porcine IgG Ab was detected in naïve B6 mice (median 0.9%, range 0.8-3.3%, n=3, Figure 3-4D). Untreated mice transplanted with NPI (median 16.7%, range 11.0-26.5%, n=3, Figure 3-4E) and anti-NK1.1 mAb treated mice transplanted with NPI (median 20.2%, range 13.8-42.8%, n=3, Figure 3-4F) demonstrated the presence of anti-porcine IgG Ab in their serum. These results demonstrate that anti-NK1.1 mAb treatment in both rat and NPI mouse recipients does not affect humoral immune response.

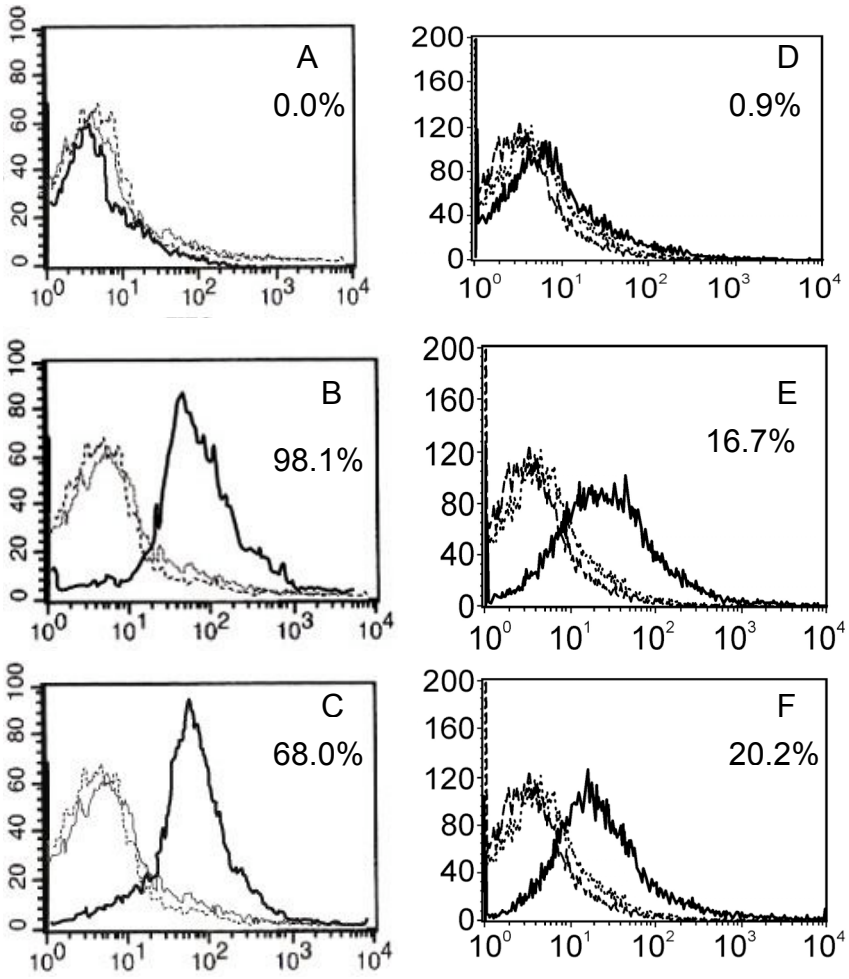

Figure 3-4: Representative mouse anti-donor IgG antibody levels in rat islet and NPI xenograft B6 mouse recipients. Donor rat spleen cells were incubated in a 1:128 dilution with serum from naïve B6 mice (A), untreated rat islet xenograft recipients (B), and anti-NK1.1 mAb treated rat islet xenograft recipients (C). Similarly, donor porcine spleen cells were incubated in a 1:128 dilution of serum from naïve B6 mice (D), untreated NPI xenograft recipients (E) and anti-NK1.1 mAb treated NPI xenograft recipients (F). Levels of bound anti-rat and anti-porcine IgG antibody were detected with a FITC-conjugated, rat adsorbed goat anti-mouse antibody using flow cytometry. In each panel, dashed (---) lines represent unstained porcine spleen cells, light grey (—) lines represent spleen cells incubated with secondary anti-mouse IgG antibody alone and solid black lines (═) represent spleen cells incubated with mouse serum and secondary antibody. Anti-rat antibody was not detected in B6 naïve mouse serum (A), while significant levels of anti-rat IgG was detected in untreated (range 98.0-98.2%, n=2, B) and anti-NK1.1 mAb treated (median 68.0%, range 10.7-100.0%, n=3, C) rat islet xenograft recipients. Similarly, anti-porcine IgG Ab was not detected in B6 naïve mouse serum (D), while ant-porcine IgG Ab was detected in untreated (median 16.7%, range 11.0-26.5%, n=3, E) and anti-NK1.1 mAb treated (median 20.2%, range 13.8-42.8%, n=3, F) NPI xenograft recipient mice.

3.3.4 $CD4^+$ AND $CD8^+$ T CELL POPULATIONS ARE UNAFFECTED BY ANTI-NK1.1 MONOCLONAL ANTIBODY TREATMENT

The finding that anti-donor Ab production occurs in both rejected and protected islet xenograft recipients suggests that $CD4^+$ T cell activation, a requisite process for antibody production, is not affected by anti-NK1.1 mAb treatment. While activation of NK cells results in an increase in cytotoxic activity (7), another role of NK cells is to exert substantial paracrine effects on T cell populations from secreted IFNγ (19, 20). Therefore, using flow cytometry we determined the effect of anti-NK1.1 mAb treatment on $CD4^+$ and $CD8^+$ T cell populations in spleen cells of B6 mice transplanted with rat islets and NPI.

We found that there were no significant changes in the $CD4^+$ T cell population compared to naïve B6 mice (23.9 ± 1.5%, n=6) in both untreated, rat islet transplanted B6 mice (19.4 ± 1.6%, n=6, p=0.06) at >100 days post-transplantation, nor in anti-NK1.1 mAb treated, rat islet xenograft B6 mouse recipients (24.4 ± 2.2%, n=4, p=0.11) at >100 days post-transplantation. Meanwhile, the $CD8^+$ T cell population, compared to naïve B6 mice (16.9 ± 1.1%, n=6), was significantly decreased in both untreated, rat islet xenograft recipients (12.2 ± 0.7%, n=6, p=0.01) and anti-NK1.1 mAb treated, rat islet xenograft recipients (13.0 ± 1.1%, n=4, p=0.04). However, there was no significant difference in $CD8^+$ T cell populations between the untreated and treated rat islet xenograft recipients (p=0.52, Figure 3-5).

A similar trend was observed in B6 mice transplanted with NPI xenografts. The $CD4^+$ T cell population compared to naïve B6 mice (23.9 ± 1.5%, n=6) was not significantly changed in untreated NPI xenograft recipients (24.0 ± 2.1%, n=4, p=0.97) nor in anti-NK1.1 treated NPI xenograft recipients (20.0 ± 2.8%, n=6, p=0.25).

Likewise, the $CD8^+$ T cell population, compared to naïve B6 mice (16.8 ± 1.1%, n=6), was decreased in untreated NPI xenograft recipients (10.7 ± 2.1%, n=4, p=0.05) and in anti-NK1.1 mAb treated NPI xenograft recipients (10.3 ± 1.3%, n=6, $p<0.01$). However, no significant difference in $CD8^+$ T cell population was noted between untreated and anti-NK1.1 mAb treated NPI xenograft recipients (p=0.89, Figure 3-5). Taken together, these results demonstrate that while anti-NK1.1 mAb treatment has drastically different effects on rat and porcine islet xenograft survival, this difference is not associated with significant changes in the systemic $CD4^+$ and $CD8^+$ T cell populations.

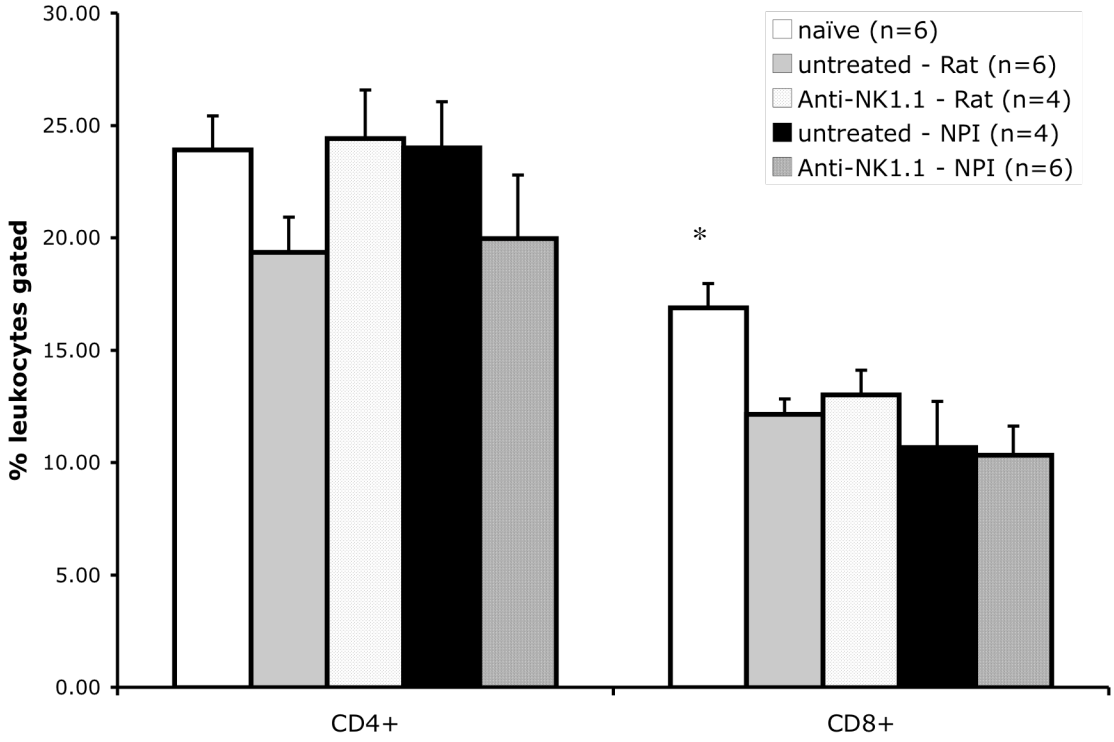

Figure 3-5: $CD4^+$ and $CD8^+$ T cell populations in spleens of B6 mice transplanted with rat islets or NPI. Splenocytes were collected from mice and $CD4^+$ and $CD8^+$ T cell populations were determined using flow cytometry. In both rat islet and NPI transplanted B6 mice, no significant change in $CD4^+$ T cell population was observed whether mice were left untreated or treated with anti-NK1.1 mAb. In both rat islet and NPI transplanted B6 mice, $CD8^+$ T cell population was significantly decreased compared to naïve B6 mouse levels. However, no significant difference was observed between untreated and anti-NK1.1 mAb treated mouse recipients.

* $p<0.05$: naïve vs. untreated rat islet; naïve vs. anti-NK1.1 mAb treated rat islet; naïve vs. untreated NPI; and naïve vs. anti-NK1.1 mAb treated NPI

3.4 DISCUSSION

Inhibiting rejection of islet xenografts in T1DM patients is complex due to the presence of both a xenoreactive immune response to the islet xenograft and a potential autoimmune reactivity to xenogeneic islet cells. It is believed that NK cells are involved in both the autoimmune development and the xenoreactive response to islet grafts (6, 21, 22). Therefore, inhibiting NK cell activity in islet xenotransplantation may help to suppress both xenoreactive and potential autoimmune responses. We examined the xenoreactive immune response in isolation of the autoimmune response and found that NK cells are important in concordant rat islet xenograft rejection and their depletion serves as an effective means of prolonging graft survival.

A previous report indicated that human NK cells exhibit an antibody-dependent cell mediated cytotoxic (ADCC) reactivity against porcine cells, including fetal porcine islet cells (22), thereby implicating a direct cytotoxic role of NK cells in the rejection of islet grafts. However, also implicated in this rejection process is a role for both $CD4^+$ T cells and B cells as these two cell populations are required for producing antibodies for the ADCC response. We have shown here that anti-NK1.1 mAb administration promotes long-term islet xenograft survival (Table 3-1) without significantly affecting the production of xenoreactive antibodies (Figure 3-4C) or $CD4^+$ T cell or $CD8^+$ T cell populations (Figure 3-5) required for the ADCC response. However, administration of the continuous anti-NK1.1 mAb regimen does reduce the NK cell population in PBMCs (Figure 3-2) and this is correlated with prolonged concordant islet xenograft survival. Our data, therefore, suggest that concordant islet xenograft rejection is independent of the humoral response alone and that the targeted elimination of the NK cell population is

sufficient to inhibit the rejection of concordant islet xenografts.

Yi *et al.* have further determined a definite role for $CD4^+$ T cells in islet xenograft rejection as they have found that NK cells and macrophages are both recruited to the islet xenograft site through a mechanism that is dependent on the production of IFNγ from $CD4^+$ T cells (6). Furthermore, in the absence of IFNγ, the process of rat islet xenotransplant rejection appears to be aborted (23, 24). Therefore, although the action of $CD4^+$ T cells is required and sufficient for the rejection of islet xenografts (25) they act by recruiting the direct cellular mediators of rejection. Our data show that NK cells act as the cellular mediator of graft rejection and reducing their numbers with the continuous administration of anti-NK1.1 mAb can inhibit concordant rat islet xenograft rejection. Meanwhile, despite the fact that macrophage are recruited to the rat islet xenograft site (6), they are not sufficient for directly killing concordant islet xenografts nor does their depletion prevent concordant islet xenograft rejection (14).

In contrast, our findings that continuous administration of anti-NK1.1 mAb does not prevent the rejection of NPI xenografts (Table 3-1) lends further support to the belief that NK cells play a minimal role in discordant islet xenograft rejection. Previous literature has demonstrated that continuous administration of anti-NK1.1 mAb did not prevent the rejection of fetal porcine islet xenograft rejection as evidenced by histologic xenograft rejection (5), nor prolong function of adult porcine islet xenograft (16). Unlike the concordant rat islet xenograft model (14), it appears that discordant models of islet xenotransplantation rejection are more dependent on macrophage (14, 26). Fetal, neonatal and adult porcine islet sources for xenotransplantation have been studied extensively in small animal models. However, pre-clinical non-human primate models

have focused largely on neonatal (3) and adult (4, 27) porcine islet sources for transplantation. While it is unclear at present time which source of porcine islets will be used if porcine islet xenotransplantation proceeds to the clinical arena, studies to date, including our results suggest in a mouse model, NK cells play a minimal role in porcine islet xenograft rejection.

It is unclear, however, whether the mechanism of porcine to human islet xenotransplantation more closely resembles concordant or discordant islet xenograft rejection. Pre-clinical trials of porcine islet xenotransplantation in non-human primates provide a recipient model closer in phylogeny to humans. In a study by Kirchhoff and colleagues it was noted that early cellular rejection of adult porcine islets in rhesus macaques is characterized by neutrophils, $CD4^+$ T cells, $CD8^+$ T cells and macrophages (28). Notably, however, in untreated and immunosuppressed macaques with rejected porcine islet xenografts, $CD56^+$ NK cell staining were not detected in acutely rejecting islet xenografts up to 72 hours post-transplantation (28), nor in grafts that had undergone cell-mediated rejection at 24 and 45 days post-transplantation (4). While NK cells comprise only 2-18% of circulating lymphocyte cells in humans (7), it appears that human NK cells are xenoreactive against porcine cells (22). Kumagai-Braesch and colleagues also demonstrated that human NK cells are cytotoxic against both human and porcine cells and lack species specificity (22).

NK cells may in fact play paradoxical roles in the establishment of islet xenografts. While our data suggests that NK cells play a role in concordant islet xenograft rejection, $NK1.1^+$ cells have also been shown to play an important role in enabling islet graft survival induced by a combination of co-stimulatory blockade by anti-

CD154 and anti-LFA-1 mAbs in both islet allograft (13) and concordant islet xenograft (29) models. The mechanism by which NK cells confer islet graft protection induced by anti-CD154 and anti-LFA-1 mAbs is perforin-granzyme dependent (13) and contribute to transplant tolerance induction by killing donor antigen-presenting cells (30). Therefore, targeting NK cells to prevent islet xenograft rejection, may have a detrimental effect on the ability to prolong islet xenograft survival and induce tolerance by way of T cell co-stimulatory blockade.

Asea and Stein-Streilein have reported that the NK1.1 receptor is not only found on NK cells but also on a portion of a small NKT cell population and that in addition to NK cell death, NK1.1 ligation results in NKT cell activation and the subsequent release of IL-4 (31). Another report has indicated that CD4+ NKT cells play an immunoregulatory role in islet xenotransplantation and has shown this cell population to be essential for acceptance of rat islet xenografts in mice (32). It is, therefore, possible that the benefit of continuous anti-NK1.1 mAb administration lies in its ability to promote a protective islet xenograft environment through both the immunoregulatory actions of NKT cells and the elimination of cytotoxic NK cells.

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CHAPTER FOUR

COMBINATION OF CTLA4-Ig AND MONOCLONAL ANTIBODY THERAPY PREVENTS THE REJECTION OF NEONATAL PORCINE ISLET XENOGRAFTS IN IMMUNE-COMPETENT MICE

4.1 INTRODUCTION

Islet transplantation has been demonstrated to be an effective treatment for brittle type 1 diabetic patients. However, in order for this treatment to become more widely adopted, especially amongst young type 1 diabetics prior to the onset of secondary complications, anti-rejection treatments which avoid the use of chronic immunosuppressive drugs must be developed. Chronic immunosuppressive drugs carry the risk of cancer and infection (1, 2). Additionally, many immunosuppressive drugs are both diabetogenic and harmful to islets. The use of corticosteroids causes insulin resistance (3), while cyclosporine and tacrolimus result in decreased insulin secretion and impaired insulin sensitivity (1-3). Therefore, there is an obvious advantage to avoiding or minimizing the need for chronic immunosuppressive drugs. However, alternative therapies must be developed to overcome the significant barrier of immune rejection of islet transplantation in type 1 diabetic patients.

Immunological tolerance is a physiological mechanism intended to limit immune responses to self-antigens. Harnessing the immune system's ability to differentiate and protect specific tissues is one strategy which could limit the need for chronic immunosuppressive drugs in islet transplant recipients. Two types of immune tolerance mechanisms exist: central tolerance and peripheral tolerance. Central tolerance involves the clonal deletion of self-reactive pre-T cells in the thymus (4). Peripheral tolerance, on the other hand, can be achieved by different mechanisms. These include T cell deletion through activation induced cell death, "ignorance" of self-antigens, induction of T cell anergy and active suppression of auto-reactive T cells by regulatory T cells (5).

A promising strategy to prevent islet xenograft rejection and facilitate tolerance induction is to block costimulatory signals to inhibit T cell activation. T cells play a central role in the rejection of islet xenografts (6, 7) and is dependent on two signals for activation. The first signal involves antigen-specific signaling through the T cell receptor (TCR). The second co-stimulatory signal is antigen-independent and occurs as various co-stimulatory molecules on the surface of T cells interact with their ligands on antigen presenting cells (APCs). If a T cell engages with an APC to receive the antigen-dependent signal 1 in the absence of adequate co-stimulation, the T cell can become anergic and undergo apoptosis (8-11).

A major T cell costimulatory pathway involves the CD28 molecule which is constitutively expressed on T cells. CD28 interaction with the B7 family of costimulatory ligands (B7-1/CD80 and B7-2/CD86) on APCs is essential for initiating antigen specific T cell responses, upregulating cytokine expression and promoting T cell expansion and differentiation (8). CTLA4 is a coinhibitory molecule transiently expressed on the surface of T cells which, similar to CD28, also binds to B7-1 and B7-2 but with higher affinity. By competitively binding the B7 family of costimulatory ligands, expression of CTLA4 effectively inhibits the T cell CD28:B7 costimulatory pathway (8). The development of a soluble CTLA4-Ig fusion protein (12) has been utilized as a therapeutic agent to prevent costimulation through the CD28:B7 pathway. This strategy has been shown to be effective in preventing rejection of human to mouse (13) and porcine to rat (14) models of islet xenotransplantation.

A related molecule to CD28, inducible costimulator (ICOS), is an additional costimulatory molecule involved in T cell activation. Although structurally similar to

CD28, ICOS does not bind to B7-1 or B7-2 but rather B7-related protein-1 (B7RP-1). B7RP-1, expressed on macrophage and dendritic cells, when bound to ICOS results in T cell proliferation and differentiation (15-17). As its name implies, ICOS is not expressed constitutively on naïve T cells but its expression is induced on activated T cells, suggesting that it is important in regulating antigen-specific activation of T cells. Targeting ICOS with anti-ICOS monoclonal antibody (mAb) has been shown to be effective in preventing islet allograft rejection when combined with anti-CD40L (18), CTLA4-Ig or rapamycin (19). In the field of islet xenotransplantation, the combination of CTLA4-Ig and anti-ICOS mAb has been demonstrated to have some effect in the prevention of rat islet to mouse xenotransplantation (20), however, the effectiveness of this combination has yet to be examined in porcine islet xenotransplantation.

Once activated, T cells express CD154 (CD40L) on their surface. CD40, the receptor for CD154, is found on APCs and when activated augments the ability of APCs to present antigen, express co-stimulatory molecules and secrete IL-2 (21). Targeting CD40:CD154 interaction has served as a major therapeutic target for prolongation of both allograft and xenograft survival (22-25). The combination of anti-CD154 mAb and donor-specific transfusion is effective in promoting rat and porcine islet xenograft survival in mice (26, 27), while the combination of anti-CD154 mAb and anti-lymphocyte function-associated antigen-1 (LFA-1) mAb is extremely effective in prolonging NPI xenografts in mice (28). Anti-CD154 mAb as a component of anti-rejection therapy in non-human primates has also been shown to be highly effective at prolonging porcine islet xenograft survival (29, 30). A major side-effect of anti-CD154 mAb therapy, however, is the development of thromboembolic complications (31).

Islet allograft prolongation has additionally been achieved by targeting the co-stimulatory molecule CD45 (32), a transmembrane protein phosphatase involved in T cell activation (33). While the ligand to CD45 remains to be identified, studies in CD45 deficient cell lines have suggested that CD45 is a positive regulator of antigen receptor signaling (33). CD45RB, a restricted isoform of CD45, has been demonstrated to be an effective target of mAb therapy in islet allotransplant prolongation and tolerance induction (34-36). However, only modest results are seen when given as monotherapy in a porcine islet xenograft model. Rayat and Gill demonstrated that anti-CD45RB mAb administration resulted in prolonged NPI xenograft survival in only 3 of 8 mice (28). When combined with anti-LFA-1 mAb, however, anti-CD45RB mAb resulted in long-term NPI xenograft survival in 8 of 12 mice (28). Therefore despite the fact that the mechanism of anti-CD45RB mAb therapy has yet to be fully elucidated, it remains an attractive target in the prevention of NPI xenograft rejection given the potential to induce transplant tolerance (34, 37).

Disruption of cell adhesion/homing receptors through the administration of mAb therapy is another effective means by which allograft and xenograft prolongation has been achieved. LFA-1 is a β2 integrin found on the surface of T cells that plays a role in cell adhesion and leukocyte homing (38). LFA-1 interacts with its ligands, intercellular adhesion molecule-1 (ICAM-1) and intercellular adhesion molecule-2 (ICAM-2), which are found on endothelial cells and results in leukocyte extravasation. ICAMs are also found on APCs and their interaction with LFA-1 on T cells strengthens the association of the T cell receptor and MHC molecules (38). Short-term blockade of LFA-1 with mAb therapy is highly effective in promoting long-term islet allograft survival and tolerance

(39-42). When administered as a monotherapy, anti-LFA-1 mAb results in modest long-term porcine islet xenograft survival, however, when combined with anti-CD154 mAb this combined therapy is highly effective in inducing NPI xenograft survival and tolerance (28, 43). Efalizumab, a humanized IgG1 form of anti-LFA-1 mAb has recently been approved for use in 35 countries, including Canada, for the treatment of moderate to severe psoriasis (44, 45). Minor side-effects associated with Eflizumab administration include headache, chills, fever, nausea, vomiting and myalgia while the more serious side-effect of immune mediated hemolytic anemia may also occur. With phase IV clinical trials demonstrating the safety of administering this agent to human patients, translation of Efalizumab to the treatment of islet transplant recipients is facilitated.

Using combination therapies targeting distinct co-stimulatory pathways, attempts have been made at eliminating the use of immunosuppressive drugs and potentially avoiding the use of long-term anti-rejection therapy altogether. Safley et al demonstrated that combining CTLA-4Ig and anti-CD154 mAb administration delayed immunohistochemical evidence of rejection in encapsulated adult porcine islet grafts in autoimmune prone diabetic NOD mice (46). While monotherapies of anti-CD45RB, anti-CD154 and anti-LFA-1 mAbs result in long-term graft survival in a minority of non-autoimmune B6 mice (28), combining co-stimulatory blockade dramatically improves NPI xenograft survival. By combining anti-LFA-1 mAb with either anti-CD45RB mAb or anti-CD154 mAb, long-term NPI xenograft survival was achieved in 8 of 12 and 12 of 14 mice respectively (28). To date, co-stimulatory blockade with the use of anti-CD154 has proven to be highly effective in small animal and non-human primate models of islet xenotransplantation. However, due to the thromboembolic complications associated with

use of this mAb, it is imperative that development of alternative combinations of treatments be developed avoiding the use of anti-CD154 mAb. As a result, short-term administration of monoclonal antibodies to distinct co-stimulatory pathways provides a promising strategy to prevent porcine islet xenograft rejection while avoiding the side effects associated with administration of long-term immunosuppressive drugs.

In this study we examined the use of CTLA4-Ig, anti-LFA-1 mAb, anti-CD45RB mAb and anti-ICOS mAb in the prevention of NPI xenograft rejection in a non-autoimmune B6 mouse model. Our findings demonstrated that administered as a monotherapy, CTLA4-Ig, anti-CD45RB mAb and anti-ICOS mAb had little effect in preventing the rejection of NPI xenografts, while anti-LFA-1 mAb administered alone prolonged NPI xenograft survival in 67% of mice. When CTLA4-Ig was administered in combination with anti-LFA-1 mAb there was no improvement in NPI xenograft survival. However, CTLA4-Ig administered in combination with anti-CD45RB mAb or anti-ICOS mAb resulted in prolonged NPI xenograft survival and demonstrate that short-term blockade of multiple T cell co-stimulatory pathways is efficacious in promoting long-term NPI xenograft survival.

4.2 MATERIALS AND METHODS

4.2.1 ANIMALS

One to 3 day old Landrace-Yorkshire neonatal pigs of either sex (1.5-2.0 kg, University of Alberta farm, Edmonton, AB, Canada) were used as islet donors. Six to 8 week old male C57BL/6J (B6, H-2^b, Jackson Laboratory, Bar Harbor, ME, USA) and immune-deficient C57Bl/6-rag1 $^{tm1/mom}$ (B6 *rag* -/-, H-2^b, Jackson Laboratory) mice were

used as recipients. All animals were fed standard laboratory food and cared for according to the guidelines established by the Health Sciences Animal Policy and Welfare Committee at the University of Alberta.

4.2.2 NEONATAL PORCINE ISLET ISOLATION

NPI were isolated as previously described (47). Briefly, neonatal pigs were anesthetized and subjected to laparotomy and exsanguinated by means of abdominal aortic catheterization. The pancreas was surgically removed under sterile conditions and placed in cold Hank's Buffer Salt Solution (HBSS, Sigma, St Louis, MO, USA). Sterile scissors were used to cut the pancreas into 1-2 mm pieces and then digested with 2.5 mg/ml collagenase (Sigma). Digested tissue was filtered through a 500 μm nylon screen then cultured for 7 days in Ham's F10 medium containing 10 mmol/l glucose, 50 μmol/l isobutylmethylxanthine (ICN Biomedicals, Montreal, PQ, Canada), 0.5% bovine serum albumin (fraction V, radioimmunoassay grade; Sigma), 2 mmol/l L-glutamine, 3 mmol/l $CaCl_2$, 10 mmol/l nicotinamide (BDH Biochemical, Poole, England), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C, 5% CO_2, 95% air. A complete media change was performed on culture days 1, 2, 4 and 6.

4.2.3 EXPERIMENTAL DESIGN

Recipient mice were rendered diabetic by intraperitoneal (i.p.) injection of streptozotocin 180mg/kg for B6 and 175mg/kg for B6 *rag* -/- (Sigma) 4-6 days before transplantation. Mice with two consecutive non-fasting blood glucose levels of ≥17

mmol/l were used as recipients. Two thousand NPI equivalents were transplanted under the left kidney capsule of diabetic B6 and B6 *rag -/-* mice.

Anti-rejection therapies were administered into the peritoneum with the following protocol: 1) CTLA4-Ig (mouse IgG2a, Bio Express Inc., West Lebanon, NH, USA) 50 μg on day -1 pre-transplantation and on days 1, 3, 5, 7, 9, 11, 13, and 15 post-transplantation; 2) anti-LFA-1 mAb (generously provided by Dr. R. Gill, University of Alberta) 200 μg on the day of transplantation and on days 1, 7, 14 post-transplantation; 3) anti-CD45RB mAb (mouse IgG2a, Bio Express Inc.) 300 μg on day -1 pre-transplantation and 100 μg on the day of transplantation and days 1, 2, 3, 4, 5 post-transplantation; or 4) anti-ICOS mAb (mouse IgG2a, Bio Express Inc.) 200 μg on the day of transplantation and daily until day 14 post-transplantation.

B6 mouse recipients were treated with intra-peritoneal administration of 1) CTLA4-Ig and anti-LFA-1 mAb; 2) CTLA4-Ig and anti-CD45RB mAb; or 3) CTLA4-Ig and anti-ICOS mAb at the same dosage and frequency as monotherapy agents. Control mice were treated with IgG2a Isotype antibody (Rat IgG2a; Bio Express Inc.) at the highest dosage combination equivalent to CTLA4-Ig and anti-LFA-1 mAb therapy. B6 *rag -/-* mouse recipients received no treatment.

Islet graft function was monitored two times a week by blood glucose measurements. Graft function was defined as blood glucose values ≤8.4 mmol/l whilst graft rejection was defined as the first of three consecutive days of blood glucose values >10 mmol/l and was further confirmed by histological analysis of the islet graft. Islet graft function was confirmed by the ability to reverse diabetes in B6 *rag -/-* mouse recipients. Furthermore, in recipients with long-term (>100 days) graft survival, return to

hyperglycemia following nephrectomy of the graft-bearing kidney established that normoglycemia was graft-dependent.

4.2.4 IMMUNOHISTOLOGICAL ANALYSIS

Graft-bearing kidneys were harvested following rejection or >100 days post-transplantation and fixed in 10% buffered formalin solution and embedded in paraffin. Insulin staining was performed on 5 μm sections. Slides were incubated for 30 min with guinea pig anti-insulin antibody (1:1000 dilution, Dako Laboratories, Mississauga, ON, Canada) followed by a 20 min incubation with biotinylated goat anti-guinea pig IgG secondary antibody (1:200 dilution, Vector Laboratories, Burlington, CA, USA). Slides were incubated with avidin-biotin complex/horseradish peroxidase for 40 min (Vector Laboratories). Positive reaction was determined using 3,3-diamino-benzidine-tetrahydrochloride (DAB), a substrate for horseradish peroxidase (BioGenex, San Ramon, CA, USA). Slides were counterstained with Harris' hematoxylin and eosin. Negative staining was achieved by omitting the primary antibody.

Specimens for immunohistochemical analysis of immune cells within the graft were immersed in optimal cutting temperature (OCT) compound (Ted Pella Inc, Redding, CA, USA) and snap frozen at -80° C. Five μm thick cryopreserved sections of tissue were air dried for 10 min then fixed in acetone for 3 min at 4° C. Cryopreserved sections were then washed in PBS and subsequently incubated in 2% FBS/PBS for 20 min to avoid non-specific binding. Endogenous biotin or biotin-binding proteins within the sample were blocked using an avidin/biotin blocking kit (Vector Laboratories). Rat anti-mouse CD4 antibody (1:100, BD Pharmingen, Mississauga, ON, Canada), rat anti-mouse

CD8 antibody (1:100, BD Pharmingen) or rat anti-mouse CD11b antibody (1:500, BD Pharmingen) was applied to each tissue sample for 30 min at room temperature. Subsequently, biotinylated rabbit anti-rat IgG secondary antibody (1:200, Vector Laboratories) was applied for 20 min at room temperature. Staining was developed with ABC/HP and DAB as previously described and cryopreserved sections were counter-stained with Harris' hematoxylin.

4.2.5 DETECTION OF MOUSE ANTI-PORCINE ANTIBODIES

Peripheral blood samples were collected from recipient and naïve B6 mice and centrifuged at 10,000 rpm for 10 min to isolate the serum. Spleen cells (1×10^6) from donor Landrace-Yorkshire neonatal pigs were incubated in 1 ml of a 1:128 dilution of serum from recipient mice transplanted with NPI for 1 hour at 37˚C (5% CO_2, 95% air). Spleen cells were then washed with 1x PBS and incubated with a 1:200 dilution of FITC-conjugated rat adsorbed goat anti-mouse IgG (Southern Biotechnology Associates Inc., Birmingham, AL, USA) for 1 hour at 4 ˚C. Spleen cells were then washed two times in 1x PBS and resuspended in 500 μl of FACS buffer (1x PBS, 2% FBS). The percentage of NPI spleen cells bound to mouse antibody was determined from fluorescence histograms on a BD FACS Calibur flow cytometry machine (BD Biosciences, Mississauga, ON, Canada) after gating on viable lymphocytes. Control experiments included donor spleen cells stained with either recipient mouse serum omitted or secondary antibody omitted.

4.2.6 STATISTICAL ANALYSIS

Statistical differences in graft survival between groups was determined using Fisher's exact test using SPSS statistical software, version 11.5 for Windows (Chicago, IL, USA). A p-value <0.05 was considered to be statistically significant.

4.3 RESULTS

4.3.1 NEONATAL PORCINE ISLET XENOGRAFTS FUNCTION IN CHEMICALLY INDUCED DIABETIC B6 *rag -/-* MICE

The ability of isolated NPI to reverse hyperglycemia and survive long-term was confirmed by transplanting 2,000 islets under the kidney-capsule of immune-deficient B6 *rag-/-* chemically rendered diabetic by streptozotocin. All B6 *rag-/-* mouse transplant recipients achieved euglycemia at a mean time of (70.1±9.5 days; n=17). Immunohistological examination of NPI xenografts after 100 days post-transplantation revealed well granulated insulin containing β cells with no cellular infiltrate (Figure 4-1F). Therefore, these results demonstrate that NPI xenografts transplanted under the kidney-capsule can restore euglycemia in an immune deficient murine model of streptozotocin induce diabetes consistent with our previous studies (28, 47)

4.3.2 MONOTHERAPY OF ANTI-LFA-1 MONOCLONAL ANTIBODY PREVENTS NEONATAL PORCINE ISLET XENOGRAFT REJECTION BUT NOT MONOTHERAPY OF CTLA4-Ig, ANTI-ICOS OR ANTI-CD45RB MONOCLONAL ANTIBODY

We first sought to determine the efficacy of blocking a single co-stimulatory pathway or adhesion pathway using mAb monotherapy. B6 mouse recipients were transplanted with NPI xenografts and treated with CTLA4-Ig, anti-LFA-1 mAb, anti-

CD45RB mAb or anti-ICOS mAb. Control mice were treated with isotype control mAb. All control treated mice rejected their NPI xenografts with none attaining graft function (0/5, 0%; Table 4-1). Histological evidence of graft rejection showed a fibrotic graft at 100 days post-transplantation (Figure 4-1A) compared to NPIs transplanted in B6 *rag-/-* mice which demonstrate abundant insulin containing β cells at 100 days post-transplantation (Figure 4-1F). Only monotherapy of anti-LFA-1 mAb (4/6; 67%; Table 4-1) resulted in significant long-term NPI xenograft function as compared to isotype mAb treated mice (0/6; 0%; $p<0.05$; Table 4-1). Histologically, this was supported by the presence of insulin staining cells in anti-LFA-1 mAb treated recipients after 100 days post-transplantation (Figure 4-1C). In contrast, monotherapy of CTLA4-Ig (0/6; 0%), anti-CD45RB mAb (0/10; 0%) and anti-ICOS mAb (0/6; 0%) did not result in long-term survival of NPI xenografts in B6 mice (Table 4-1). Examination of rejected NPI xenografts histologically confirmed these findings as after 100 days post-transplantation anti-CD45RB mAb (Figure 4-1D) and anti-ICOS mAb (Figure 4-1E) treated mice had completely rejected the grafts. In comparison, while CTLA4-Ig mAb treated mice did not exhibit long-term graft function, an immune infiltrate persisted at the graft site at 100 days post-transplantation (Figure 4-1B). The prolonged persistence of immune cells at the NPI xenograft site suggests that while CTLA4-Ig monotherapy is not sufficient to prevent NPI xenograft rejection, it may delay the rejection process. Therefore taken together, these results demonstrate that targeting a single co-stimulatory pathway using CTLA4-Ig, anti-ICOS mAb or anti-CD45RB mAb is not sufficient to prevent the rejection of NPI xenografts in chemically induced diabetic B6 mice. However, short-term monotherapy targeting the adhesion pathway LFA-1:ICAM-1 with anti-LFA-1 mAb

is able to induce long-term NPI xenograft survival in a proportion of B6 mouse recipients.

Mice treated with monotherapy of anti-LFA mAb and CTLA4-Ig with evidence of infiltrating immune cells were further examined to determine the phenotype of these infiltrating immune cells by immunohistochemical analysis. Anti-LFA-1 mAb treated mouse recipients demonstrated minimal immune cell infiltration with the predominant cell type being $CD4^+$ T cells (Figure 4-2A, C, E). Meanwhile, CTLA4-Ig treated mice despite having rejected their islet graft, had persistent immune cell infiltrates even at 100 days post-transplantation. These late immune cell infiltrates were characterized predominantly by $CD4^+$ T cells with a central core of $CD8^+$ T cells (Figure 4-2B, D) and surrounding the islet remnants were $CD11b^+$ macrophages (Figure 4-2F). The persistence of $CD4^+$ T cells in both anti-LFA-1 mAb treated mice with long-term graft function and CTLA4-Ig treated mice with rejected grafts could potentially represent pathogenic $CD4^+$ T cells which may ultimately reject β cells in the case of anti-LFA-1 mAb treated mice or having recently rejected β cells in CTLA4-Ig treated mice. Alternatively, these $CD4^+$ T cells may represent regulatory T cells persistent in the graft that may have a role in trying to protect β cells following treatment with anti-LFA-1 mAb or CTLA4-Ig.

Table 4-1: NPI xenograft survival in monotherapy treated B6 mice. Streptozotocin-induced diabetic B6 mice were transplanted with 2000 NPI and monitored for evidence of graft rejection to and end point of 100 days post-transplantation.

Treatment	n	Graft survival*	Long-term graft function †
Isotype control mAb	5	0 x 5	0/5 (0%)
CTLA4-Ig	6	0 x 6	0/6 (0%)
Anti-LFA-1 mAb	6	0 x 2, >100 x 4	4/6 (67%)
Anti-CD45RB mAb	10	0 x 10	0/10 (0%)
Anti-ICOS mAb	6	0 x 6	0/6 (0%)

* 0 represents mice that did not achieve euglycemia

† Long-term graft function defined as euglycemia >100 days post-transplantation

1) Isotype control mAb: 50 μg on day -1 pre-transplantation and on days 1, 3, 5, 7, 9, 11, 13, and 15 post-transplantation and 200 μg on the day of transplantation and on days 1, 7, 14 post-transplantation

2) CTLA4-Ig: 50 μg on day -1 pre-transplantation and on days 1, 3, 5, 7, 9, 11, 13, and 15 post-transplantation

3) Anti-LFA-1 mAb: 200 μg on the day of transplantation and on days 1, 7, 14 post-transplantation

4) Anti-CD45RB mAb: 300 μg on day -1 pre-transplantation and 100 μg on the day of transplantation and days 1, 2, 3, 4, 5 post-transplantation

5) Anti-ICOS mAb: 200 μg on the day of transplantation and daily until day 14 post-transplantation

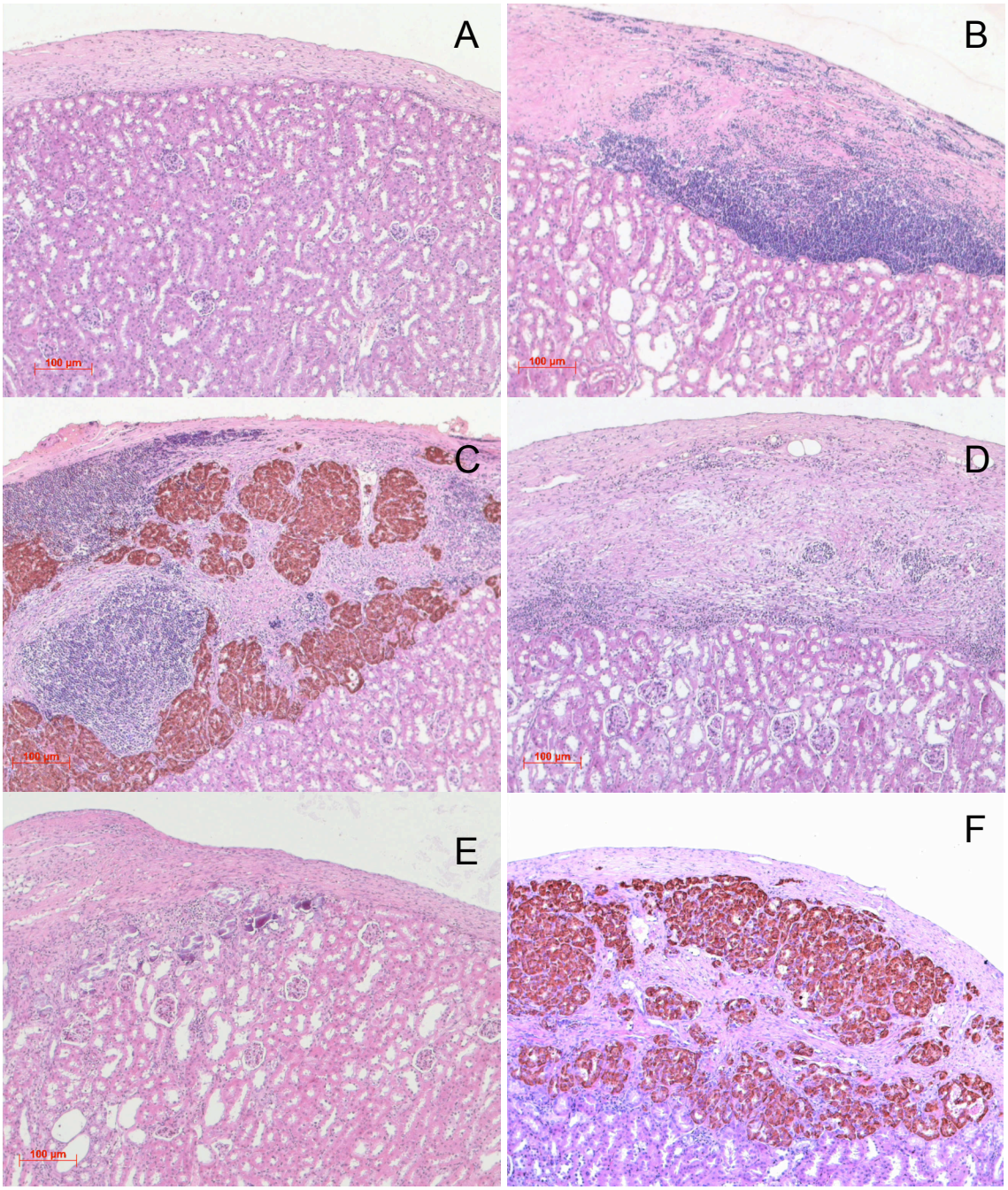

Figure 4-1: Representative insulin immunohistochemical staining of NPI xenografts in B6 mice treated with (A) IgG control (B) CTLA4-Ig (C) Anti-LFA-1 mAb (D) Anti-CD45RB mAb (E) Anti-ICOS mAb (F) Control B6 *rag*-/- mouse transplanted with NPI. 2000 NPI were transplanted under the left kidney capsule of B6 mice. Grafts were harvested at 100 days post-transplant and histologic examination for the presence of insulin positive cells reveals fibrotic grafts in IgG control, anti-LFA-1 mAb and anti-ICOS mAb treated mice. CTLA4-Ig treated mice lack evidence of insulin positive cells but an immune cell infiltrate persists at 100 days post-transplantation. Anti-LFA-1 mAb treated mice show evidence of persistent insulin positive cells with nests of infiltrating immune cells.

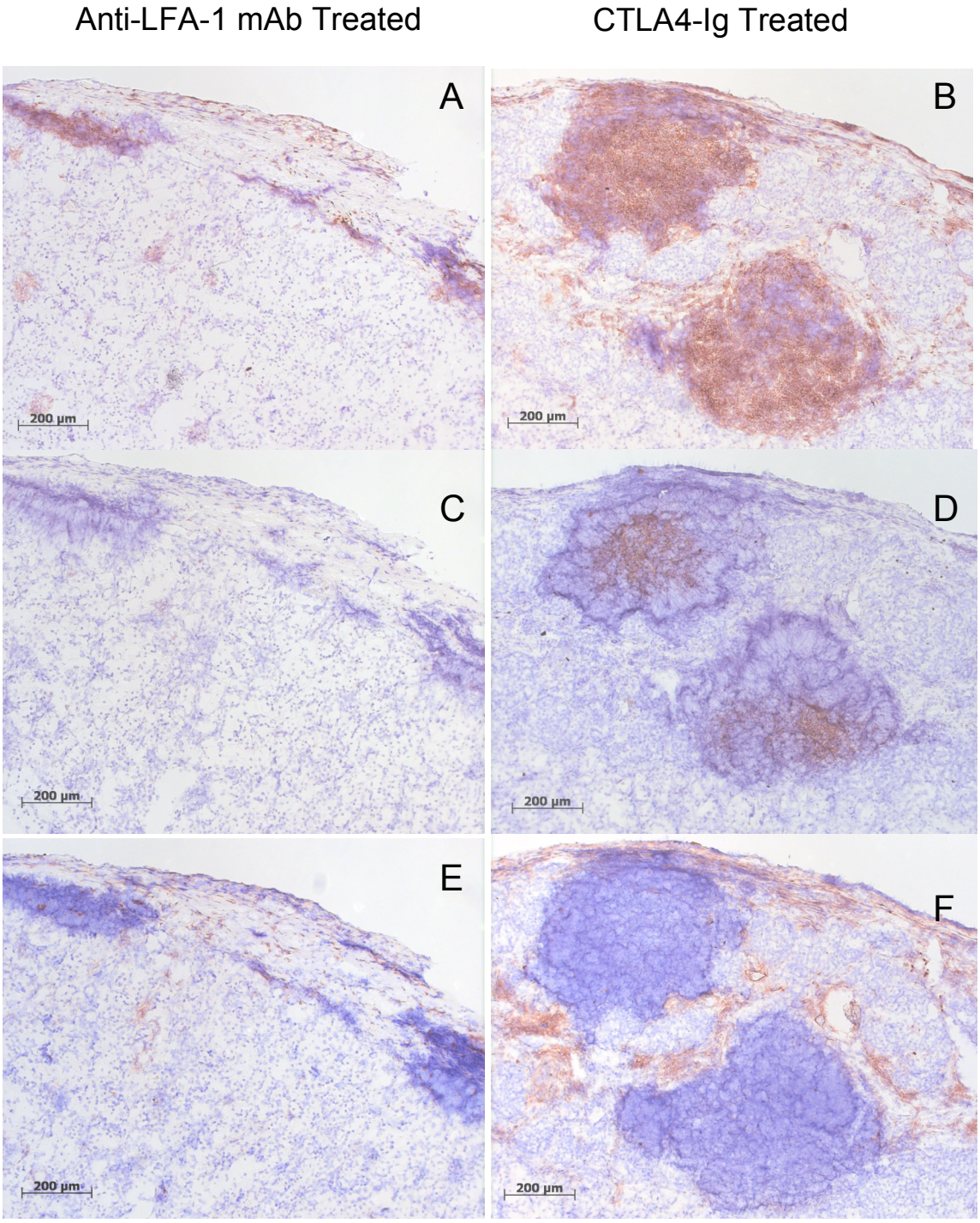

Figure 4-2: Representative immune cell immunohistochemical staining of NPI xenografts in monotherapy treated B6 mice (A,B) $CD4^+$ T cell staining (C, D) $CD8^+$ T cell staining (E, F) $CD11b^+$ staining. 2000 NPI were transplanted under the left kidney capsule of B6 mice. Infiltrating immune cells in anti-LFA-1 mAb treated mice are characterized predominantly by $CD4^+$ T cells. CTLA4-Ig treated mice demonstrate persistent immune cells characterized predominantly by $CD4^+$ T cells with a central core of $CD8^+$ T cells and peripheral $CD11^+$ macrophage cells.

4.3.3 COMBINATION OF CTLA4-Ig AND MONOCLONAL ANTIBODY CO-STIMULATORY BLOCKADE PREVENTS NEONATAL PORCINE ISLET XENOGRAFT REJECTION

While monotherapy of CTLA4-Ig was not sufficient to prevent eventual long-term rejection of NPI xenografts in B6 mice, our previous findings suggest that there may be an effect of B7:CD28 co-stimulatory blockade with CTLA4-Ig on the rejection process of NPI xenografts when grafts were examined histologically. Therefore, we set out to determine whether targeting the CD28:B7 pathway in combination with other co-stimulatory pathways or adhesion pathways could act synergistically to prevent the rejection of NPI xenografts. The combination of CTLA4-Ig and anti-LFA-1 mAb therapy resulted in long-term NPI xenograft survival in the majority of B6 mouse recipients (4/6, 67%; Table 4-2). However, the addition of CTLA4-Ig did not result in improved graft survival compared with monotherapy of anti-LFA-1 mAb (4/6, 67%; p=ns; Table 4-1). Histological examination of the graft showed that insulin staining was evident at 100 days post-transplantation in CTLA4-Ig and anti-LFA-1 mAb treated mice despite the fact that significant nests of immune infiltrating cells (Figure 4-3B) composed predominately of $CD8^+$ T cells persisted (Figure 4-4B). Therefore, simultaneous targeting the CD28:B7 co-stimulatory pathway with CTLA4-Ig and the ICAM:LFA-1 adhesion pathway with anti-LFA-1 mAb did not significantly improve NPI xenograft survival.

Administered as monotherapy, CTLA4-Ig, anti-CD45RB mAb and anti-ICOS mAb were ineffective in preventing NPI xenograft rejection (Table 2-1). However, when CTLA4-Ig and anti-CD45RB mAb were administered in combination, 33% (2/6; Table 4-2) of mouse recipients attained long-term graft survival despite the fact that this did not

reach statistical significance as compared to CTLA4-Ig monotherapy (p=0.31) or anti-CD45RB mAb monotherapy (p=0.18). These results were confirmed histologically by the presence of insulin positive β cells at 100 days post-transplantation (Figure 4-3C). Despite long-term graft survival, immune cell infiltration remained evident in the NPI xenograft at 100 days post-transplantation characterized predominantly by $CD4^+$ T cells (Figure 4-5A). When CTLA4-Ig was administered in combination with anti-ICOS mAb, 50% of mice attained long-term graft survival (3/6; 50%; Table 4-2) despite the fact that this did not reach statistical significance compared to monotherapy of both CTLA4-Ig (p=0.18) and anti-ICOS mAb (p=0.18).

Immunohistochemical analysis of NPI xenograft tissue sections was performed to determine the phenotype of infiltrating immune cells in the graft. In CTLA4-Ig and anti-LFA-1 mAb (Figure 4-4), CTLA4-Ig and anti-CD45RB mAb (Figure 4-5) and CTLA4-Ig and anti-ICOS mAb (Figure 4-6) treated mice the predominant immune cell infiltrating the graft was $CD4^+$ T cells. As was the case in immune cell infiltrates found in monotherapy anti-LFA-1 mAb and CTLA4-Ig treated mice, these $CD4^+$ T cells may potentially represent pathogenic $CD4^+$ T cells which may ultimately reject the graft or regulatory T cells which may have a role in trying to protect β cells.

Table 4-2: NPI xenograft survival in B6 mice treated with a combination of CTLA4-Ig fusion protein and mAb. Streptozotocin-induced diabetic B6 mice were transplanted with 2000 NPI and monitored for evidence of graft rejection to an end point of 100 days post-transplantation.

Treatment	n	Graft survival*	Long-term graft function †
Isotype control mAb	5	0 x 5	0/5 (0%)
CTLA4-Ig & anti-LFA-1 mAb	6	0 x 2, >100 x 4	4/6 (67%)
CTLA4-Ig & anti-CD45RB mAb	6	0 x 4, >100 x 2	2/6 (33%)
CTLA4-Ig & anti-ICOS mAb	6	0 x 3, >100 x 3	3/6 (50%)

* 0 represents mice that did not achieve euglycemia

† Long-term graft function defined as euglycemia >100 days post-transplantation

1) Isotype control mAb: 50 μg on day -1 pre-transplantation and on days 1, 3, 5, 7, 9, 11, 13, and 15 post-transplantation and 200 μg on the day of transplantation and on days 1, 7, 14 post-transplantation

2) CTLA4-Ig: 50 μg on day -1 pre-transplantation and on days 1, 3, 5, 7, 9, 11, 13, and 15 post-transplantation and Anti-LFA-1 mAb: 200 μg on the day of transplantation and on days 1, 7, 14 post-transplantation

3) CTLA4-Ig: 50 μg on day -1 pre-transplantation and on days 1, 3, 5, 7, 9, 11, 13, and 15 post-transplantation and Anti-CD45RB mAb: 300 μg on day -1 pre-transplantation and 100 μg on the day of transplantation and days 1, 2, 3, 4, 5 post-transplantation

4) CTLA4-Ig: 50 μg on day -1 pre-transplantation and on days 1, 3, 5, 7, 9, 11, 13, and 15 post-transplantation and Anti-ICOS mAb: 200 μg on the day of transplantation and daily until day 14 post-transplantation

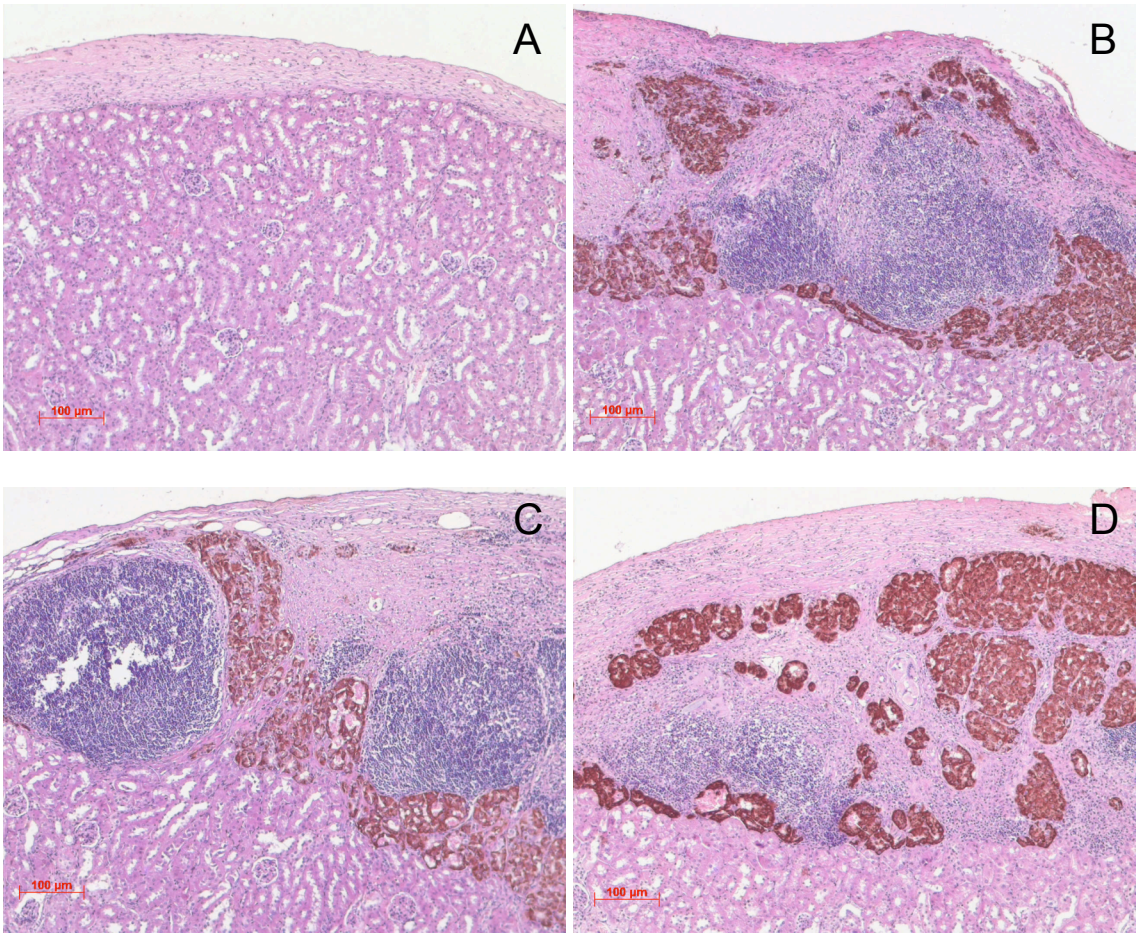

Figure 4-3: Representative insulin immunohistochemical staining of NPI xenografts in combination therapy treated B6 mice. (A) IgG control (B) CTLA4-Ig & anti-LFA-1 mAb (C) CTLA4-Ig & anti-CD45RB mAb (D) CTLA4-Ig & anti-ICOS mAb. 2000 NPI were transplanted under the kidney capsule of B6 mice. Grafts were collected at 100 days post-transplantation for immunohistochemical staining for insulin positive cells. All combinations of therapy resulted evidence of insulin positive cells long-term despite infiltration by immune cells within the graft.

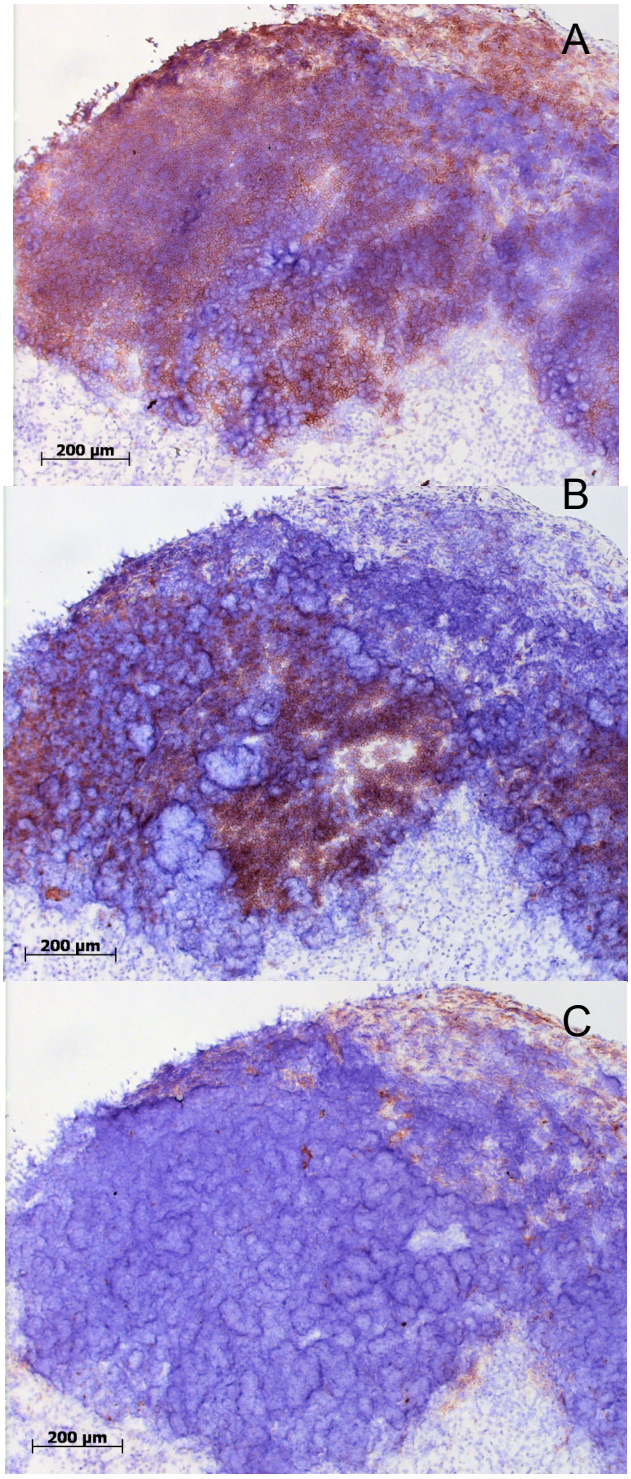

Figure 4-4: Representative immune cell immunohistochemical staining of NPI xenografts in CTLA4-Ig & Anti-LFA-1 mAb treated B6 mice (A) $CD4^+$ T cell staining (B) $CD8^+$ T cell staining (C) $CD11b^+$ staining. 2000 NPI were transplanted under the kidney capsule of B6 mice. Grafts were collected at 100 days post-transplantation and stained for immune cells. Immune cell infiltrates in CTLA4-Ig & Anti-LFA-1 mAb treated B6 mice were characterized predominately by the presence of $CD4^+$ and $CD8^+$ T cells.

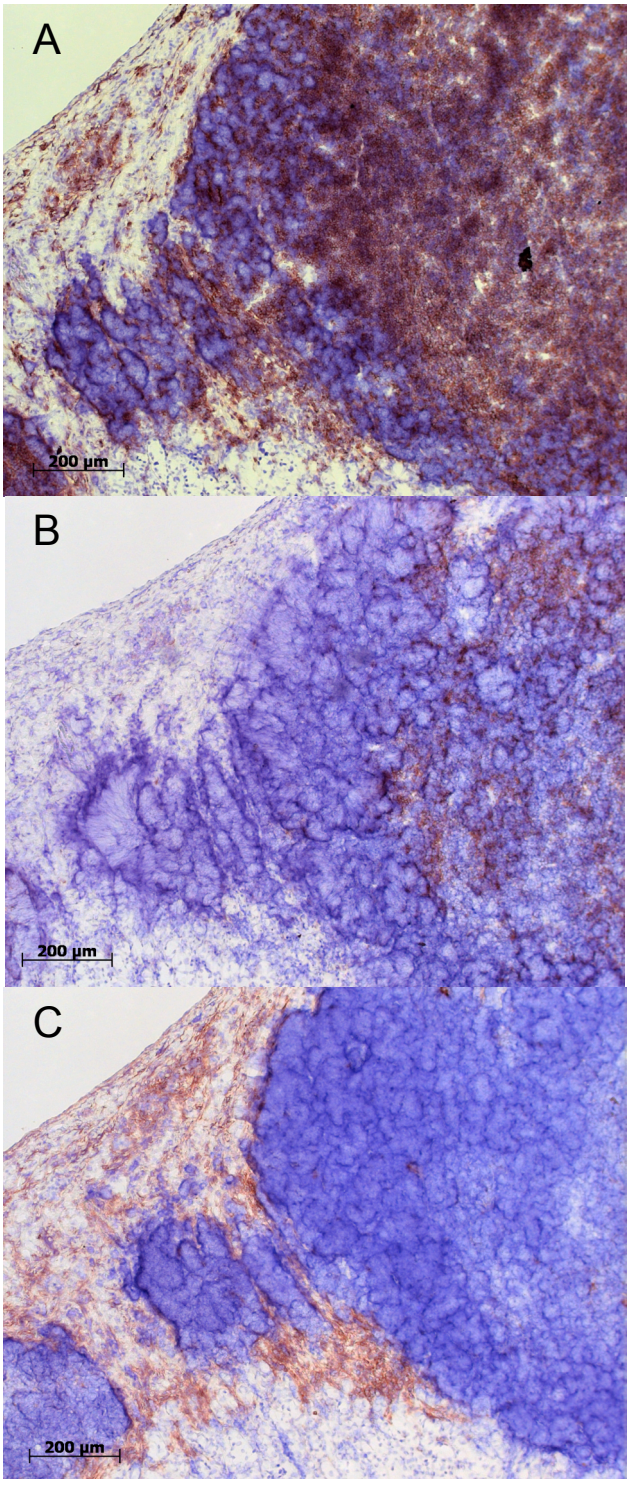

Figure 4-5: Representative immune cell immunohistochemical staining of NPI xenografts in CTLA4-Ig & Anti-CD45RB mAb treated B6 mice (A) $CD4^+$ T cell staining (B) $CD8^+$ T cell staining (C) CD11b+ staining. 2000 NPI were transplanted under the kidney capsule of B6 mice. Grafts were collected at 100 days post-transplantation and stained for immune cells. Immune cell infiltrates in CTLA4-Ig & Anti-CD45RB mAb treated B6 mice were characterized predominately by the presence of $CD4^+$ and $CD8^+$ T cells.

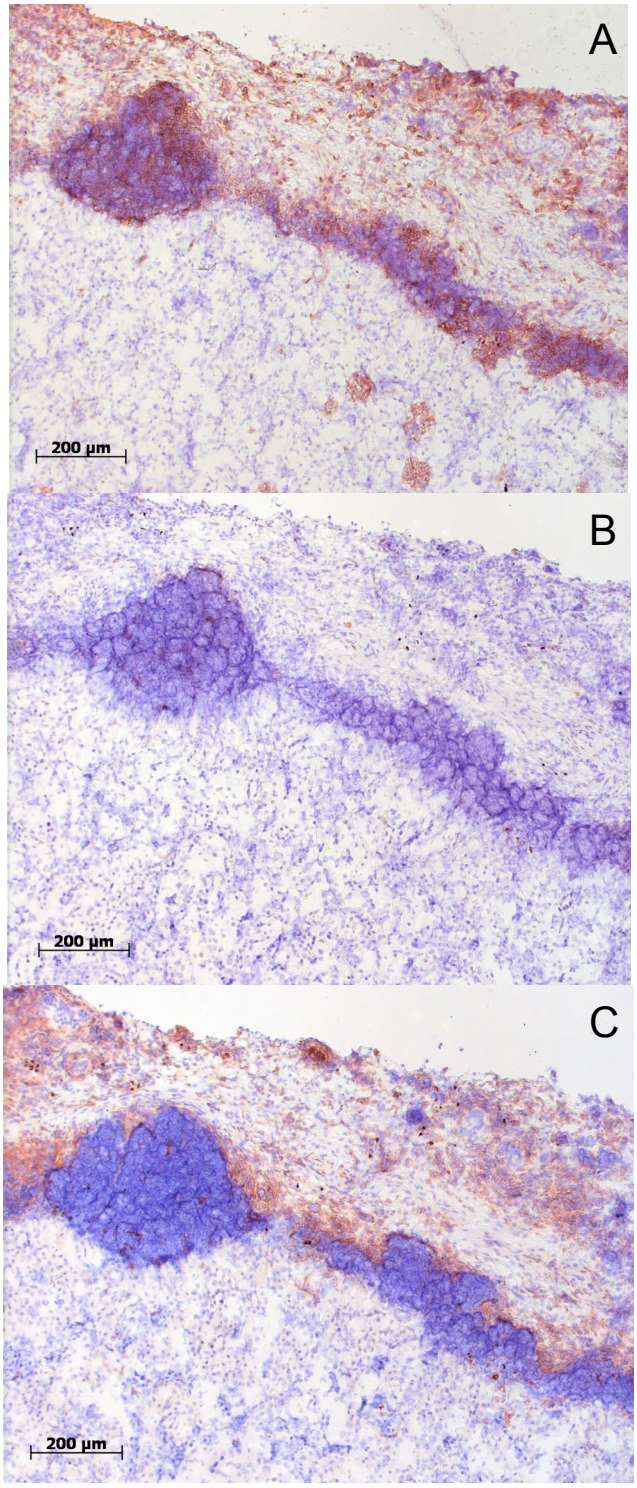

Figure 4-6: Representative immune cell immunohistochemical staining of NPI xenografts in CTLA4-Ig & Anti-ICOS mAb treated B6 mice (A) $CD4^+$ T cell staining (B) $CD8^+$ T cell staining (C) CD11b+ staining. 2000 NPI were transplanted under the kidney capsule of B6 mice. Grafts were collected at 100 days post-transplantation and stained for immune cells. Immune cell infiltrates in CTLA4-Ig & Anti-ICOS mAb treated B6 mice were characterized predominately by the presence of $CD4^+$ T cells.

4.3.4 COMBINED CO-STIMULATORY BLOCKADE PREVENTS NPI XENOGRAFT REJECTION DESPITE THE PRODUCTION OF MOUSE ANTI-PORCINE ANTIBODIES

Recipient sera was analyzed using flow cytometry for the presence of mouse anti-porcine antibody at 100 days post-transplantation. Naïve B6 mouse sera served as our control and as expected no mouse anti-porcine antibody was detected (Figure 4-7A). In comparison, B6 mouse recipients transplanted with NPI and treated with isotype control mAb, rejected their graft and at 100 days post-transplantation mouse anti-porcine antibody was detected in their serum (Figure 4-7B). Similarly, transplanted mice treated with a monotherapy of CTLA4-Ig (Figure 4-7C) or anti-CD45RB (Figure 4-7E) with rejected grafts also had detectable mouse anti-porcine antibody within their serum. However, mice treated with a monotherapy of anti-LFA-1 mAb that maintained graft function at 100 days post-transplantation had no detectable mouse anti-porcine antibody within their sera at 100 days post-transplantation (Figure 4-7D).

The combination of CTLA4-Ig and anti-LFA-1 mAb resulted in reduced mouse anti-porcine antibody in B6 mouse recipients attaining long-term graft function (Figure 4-7F). Meanwhile, the combination of CTLA4-Ig and anti-CD45RB mAb or CTLA4-Ig and anti-ICOS mAb resulted in long-term graft survival in 33% and 50% of treated mice, respectively (Table 4-2). However, in those mice that did achieve long-term graft survival combination therapy of CTLA4-Ig and anti-CD45RB mAb or CTLA4-Ig and anti-ICOS mAb did not inhibit the production of mouse anti-porcine antibody (Figure 4-7G and 4-7H).

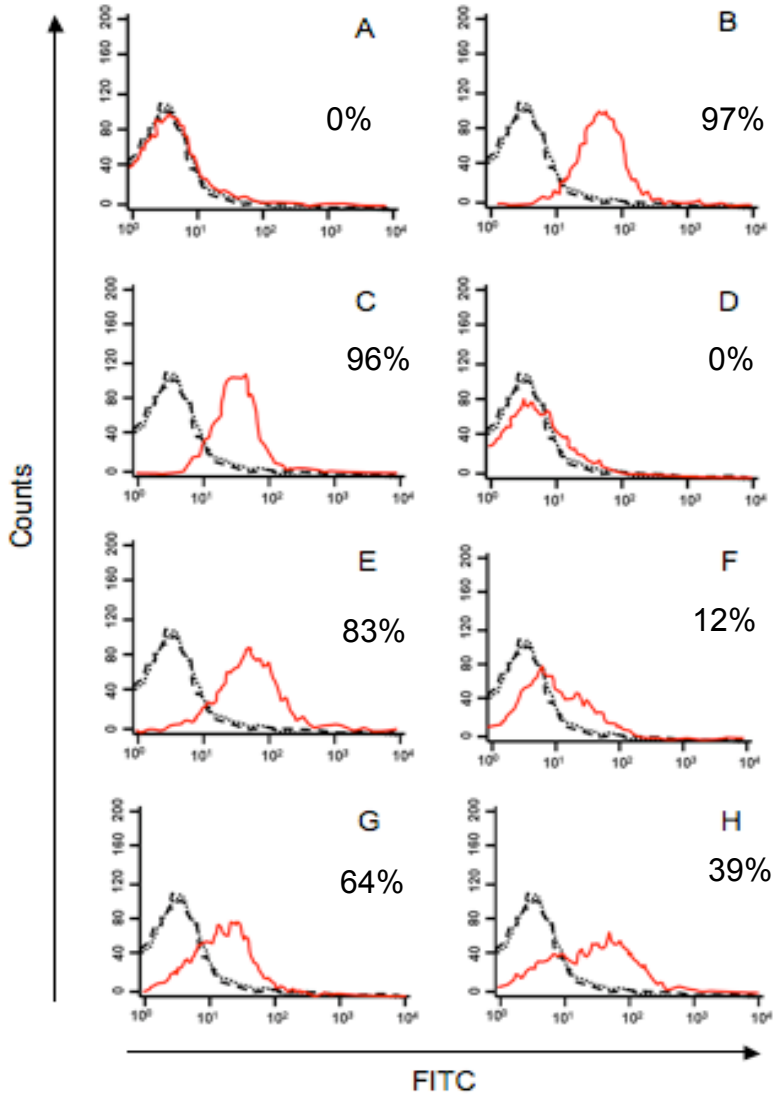

Figure 4-7: Representative mouse anti-porcine IgG antibody levels in B6 mice. (A) naïve B6 mouse (B) untreated (C) CTLA4-Ig treated (D) Anti-LFA-1 mAb treated (E) Anti-CD45RB mAb treated (F) CTLA4-Ig & anti-LFA-1 mAb treated (G) CTLA4-Ig & anti-CD45RB mAb treated (H) CTLA4-Ig & anti-ICOS mAb treated. B6 mice were transplanted with 2000 NPI and their serum was collected at 100 days post-transplantation. Mouse serum diluted 1:128 and incubated with $1x10^6$ donor porcine spleen cells. A secondary goat anti-mouse IgG antibody was administered and porcine spleen cells were analyzed by flow cytometry for evidence of bound mouse anti-porcine IgG antibody.

4.4 DISCUSSION

Indefinite NPI xenograft survival has been demonstrated by the short-term administration of T cell co-stimulatory and adhesion pathway blockade using monoclonal antibodies against CD154 and LFA-1, respectively (28). However, application of this combination of therapies is limited by the occurrence of thromboembolic complications associated with the use of anti-CD154 mAb therapy. Therefore, the objective of the present study was to identify alternative co-stimulatory and adhesion pathways to target in the prevention of NPI xenograft rejection. This study demonstrated that monotherapy targeting the co-stimulatory pathways CD28:B7, ICOS and CD45RB were not effective in preventing NPI xenograft rejection. Administration of anti-LFA-1 mAb alone, did have some efficacy in preventing NPI xenograft rejection. However, using CTLA4-Ig as a central therapy, blocking two co-stimulatory pathways was effective in promoting long-term xenograft survival in the majority of recipients. The combination of CTLA4-Ig and anti-LFA-1, did not enhance NPI xenograft survival, however, the addition of CTLA4-Ig therapy did not negate the protective effects of anti-LFA-1 mAb.

Enhanced efficacy of CTLA4-Ig and anti-CD45RB therapy may be explained by the link between CD45 activation and CTLA4. The protein tyrosine phosphatase, CD45, is recognized as a potent immunomodulatory target. However, the exact mechanisms by which this is achieved are incompletely understood. Fecteau and colleagues have demonstrated that anti-CD45RB mAb treatment results in rapid upregulation of CTLA-4 expression on T cells (37). Therefore, by simultaneously targeting CD45RB and the CD28:B7 pathway in our current study using anti-CD45RB mAb and CTLA4-Ig, perhaps we are mimicking the natural effects of CD45RB signaling.

The ICOS co-stimulatory pathway has been demonstrated to play an important role in antibody class switching and germinal centre formation (48). Specifically, in conjunction with CD40:CD154 pathway, the ICOS pathway plays a particularly important role in stimulating IgE Ab production by B cells (48). In our study we examined the effect of our combination therapies on the production of anti-porcine IgG Ab production. While monotherapies of CTLA4-Ig or co-stimulatory blockade did not prevent the production of anti-porcine IgG Ab, combination therapies with CTLA4-Ig did result in decreased anti-porcine IgG Ab production. While these results would suggest that $CD4^+$ T cell activation is inhibited by combination therapy, these results cannot also rule out possible effects on antibody class switching. Examination of levels of specific anti-porcine Ab classes would be beneficial in examining this potential role.

In summary, our findings demonstrate that short-course combination therapy with the blockade of the CD28:B7 pathway using CTLA4-Ig can improve NPI xenograft survival and confirm that activation of the CD28:B7 pathway plays an important role in islet xenograft rejection. The need for targeting multiple pathways may lie in the fact that impairment of one co-stimulatory pathway may be overcome by strongly activating other co-stimulatory pathways (49). As a result, until such time that a healthy NPI xenograft becomes established and co-stimulatory signals wane, blockade of multiple co-stimulatory pathways is likely to be necessary in order to attain long-term graft survival. While the precise mechanism by which these combinations of therapies maintain long-term NPI xenograft function has not been fully elucidated, infiltration of predominantly $CD4^+$ T cells into the graft site suggests that while T cell costimulatory blockade may not

prevent recruitment of immune cells by the graft, inhibition of anti-porcine IgG antibody responses suggest that these therapies likely act by preventing T cell activation.

Further investigation into the role of CTLA4-Ig therapy in the prevention of NPI xenograft rejection is warranted given the results of our study. Potential combinations of therapies targeting adhesion pathways using anti-LFA-1 mAb in addition to multiple co-stimulatory pathways may provide further protection to NPI xenografts. Further studies investigating the effect these combinations of therapies on auto-immune responses to NPI xenografts should also be investigated in the non-obese diabetic mouse model. Lastly, non-human primate models of NPI xenograft rejection currently require the use of continuous anti-rejection therapy (29, 30). Administration of short-course T cell costimulatory blockade would be the next step in advancing the acceptance of islet transplantation in the clinical realm.

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CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSIONS

5.1 GENERAL DISCUSSION

The outcome of patients with diabetes mellitus (DM) was drastically changed with the discovery of insulin in 1922 (1) - patients who once had a fatal outlook can now live active and productive lives. Insulin treatment, however, is not a cure for diabetes. Despite advances in insulin formulation and delivery, type 1 DM patients remain at risk for secondary vascular complications (2, 3). While insulin currently is the gold-standard for treatment of type 1 DM patients, alternative treatment options to regulate blood glucose (BG) levels in a more physiologic manner are being developed including islet transplantation (4), pancreas transplantation (5, 6), stem cell therapy (7, 8), β cell regeneration (9-11), bioartificial pancreases (12) and gene therapy (13).

Even prior to the complete understanding of the function of the human pancreas, variations of islet transplantation have been attempted (14, 15). Successful islet transplantation in humans, however, did not occur until 1989 when the research team at the University of Alberta demonstrated sustained C-peptide secretion following intra-portal islet transplantation performed concurrently with an orthotopic kidney transplantation (16). A major breakthrough in the success of clinical islet transplantation involved the development of the Edmonton protocol. Eliminating glucocorticoids from the immunosuppressive regimen and transplanting islets from two donor pancreases resulted in a drastic improvement in insulin-free rates at one year (4). Current evidence demonstrates that islet transplantation does indeed provide an advantage over current insulin therapies in controlling BG levels as demonstrated by hemoglobin A1C levels (17). Despite these successes, islet transplantation is not a viable treatment option for the majority of type 1 DM patients due to the shortage of human donor pancreatic tissue and

the need for continuous immunosuppressive drugs to prevent islet graft rejection. The development of safe and effective porcine islet xenotransplantation protocols could result in islet transplantation becoming a widely available treatment option for type 1 DM patients. While porcine islet transplantation has now been demonstrated to effectively reverse diabetes in pre-clinical non-human primate models (18, 19), the need for continuous immunosuppression remains a significant barrier in the application of this treatment.

A better understanding of the immune processes that are involved in neonatal porcine islet (NPI) xenotransplantation rejection will help to facilitate the identification of new targets for novel anti-rejection therapy and enable more specific targeting of the immune cells and molecules involved. In Chapter 2, we examined early events of NPI xenograft rejection in the absence of auto-immunity in an immune-competent B6 mouse transplant model. Our results demonstrated that early events in NPI xenograft rejection are characterized by initial infiltration of the graft site by innate immune cells such as macrophage and neutrophils. Targeting these cells which appear early in the rejection process may provide an opportunity to abort the rejection process prior to T cell activation. One particular strategy to prevent the infiltration of immune cells into the graft site may be to target the signals which draw them to the islet xenograft. In the early phases of NPI xenograft rejection we identified MIP-1α, MIP-1β, MCP-1 and RANTES as chemokines present at the transplantation site. However, using reverse-transcriptase PCR techniques, only MIP-1β was upregulated during NPI xenograft rejection.

In chapter 3 we examined the differential role of natural killer (NK) cells in concordant rat and discordant NPI xenograft models of islet xenotransplantation. We

demonstrated that NK cells play a role in the rejection of concordant rat islet xenografts in a non-autoimmune mouse model of diabetes. However, in a discordant NPI xenograft model, NK cells do not appear to play a role in rejection. Translation of experimental data in small animal models is difficult to anticipate. Transplantation of NPI into non-human primates and humans is thought to represent a concordant model of islet xenotransplantation. Therefore, these results suggest that NK cells may yet play a role in NPI rejection when applied in concordant models of xenotransplantation.

For islet transplantation to ultimately be regarded as a viable cure for diabetic patients, diabetic patients must be transplanted prior to the onset of secondary complications. In the case of type 1 DM, this may potentially involve the transplantation of juvenile patients. In order to prevent the need for long-term immunosuppression of these young patients, short-duration, tolerizing anti-rejection therapies would be the ideal treatment to prevent rejection of transplanted porcine islet xenografts. In chapter 4, B7:CD28, ICOS:ICOS-L, LFA-1:ICAM-1 and HB220 pathways are identified as potential targets in T cell activation which if blocked may prevent cell mediated rejection of porcine islet xenografts. In order to prevent NPI xenograft rejection, however, it appears that multiple co-stimulatory or adhesion pathways need to be targeted.

NPI xenograft rejection is a complex process that involves many aspects of the recipient immune system. In order for NPI xenotransplantation to become a viable treatment alternative for diabetic patients, anti-rejection therapies which carry minimal toxic risks and can be administered on a short-term basis must be available. Administering transient therapies to prevent critical steps in the process of immune reaction to NPI xenografts is one way to achieve this. However, this approach will

require further dissection of the immune mechanisms and the kinetics of NPI xenograft rejection.

5.2 CONCLUSIONS

Islet transplantation holds great promise as a potential cure for diabetic patients. In its short history, rapid advances have been made in the field of islet transplantation. Porcine islets are a readily available source of donor tissue which can meet the demands of islet transplantation. However, in this era of increased biomedical regulation, careful control of porcine donor source tissue will be essential. Given the ever increasing costs of conducting both bench and bedside research the world porcine islet research community will have to settle current controversies such as the ideal porcine islet donor age, how to best incorporate emerging donor genetic modification technologies and how to appropriately monitor for post-transplant xenozoonotic viral transmission in order for the field to continue to advance at a steady pace.

Using currently available anti-rejection therapies, transplantation of any organ is currently limited to patients suffering from the end-stages of their disease. The field of islet transplantation is helping to push the ceiling and redefine the criteria for transplant recipients. The original vision of Dr. Paul Lacy was to "transplant islets without immunosuppression early in the course of diabetes" (20). In order to convince a young, healthy type 1 DM patient to accept islet transplantation as a form of therapy for his/her chronic illness, we must have available anti-rejection therapies which compared to their daily insulin therapies are easy to administer. Secondly, these therapies must demonstrate minimal side-effects and be safe in both the short-term and in the long-term

as diabetic patients will be weighing these risks against complications from their diabetes which they will not encounter for likely another 30 or more years. Since Lacy's first description of a successful islet transplant in rats over 35 years ago, extraordinary advances have been made in procuring islets for transplantation and with advances in porcine islet xenotransplantation the shortage of islets for transplantation may soon be addressed. I believe the next breakthrough in islet transplantation will be the unravelling of our complex immune system. Using T cell costimulatory blockade we have seen glimpses of what is possible with regards to anti-rejection therapy. However, still too much is unknown about the response of the immune system to islet xenografts and a better understanding of this fascinating and complex system will be required in order to generate safe and effective treatments for diabetic patients of the future.

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