University of Alberta

The Potential of Novel Small Inhibitory Molecules to Prevent the Rejection of Neonatal Porcine Islets in Mice

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ABSTRACT

Islet transplantation is a promising treatment for type 1 diabetes, however its current clinical use is limited by the shortage of donor tissue and the need for the continuous use of harmful immunosuppressive drugs to prevent rejection of the islet transplant. The shortage of donor tissue may be remedied by the use of xenogeneic tissue, in particular neonatal porcine islets (NPI). The use of biologic agents in the form of anti-LFA-1 and anti-CD154 monoclonal antibody (mAb) therapy has been shown to effectively prevent the rejection of islet grafts without the use of immunosuppressive drugs. However, the humanized form of anti-CD154 mAb is no longer available due to the increased incidence of thromboembolic events. Thus, an alternative to anti-CD154 mAb is being sought. Small inhibitory molecules, in particular, Suramin and Direct Red 80 (DR80), have been recently shown to inhibit the CD40-CD154 interaction *in vitro*. This led us to determine the effect of Suramin and DR80 on T cell proliferation *in vitro,* and to determine whether they could prolong the survival of NPI in mice, in the presence or absence of anti-LFA-1 mAb.

Our data show that Suramin and DR80 are able to inhibit T cell proliferation *in vitro* in a dose dependent manner with minimal cytotoxicity. None of the mice treated with Suramin alone (n=6) achieved normoglycemia, and only 1/7 mice treated with suramin and anti-LFA-1 mAb did. The combination of DR80 with anti-LFA-1 mAb allowed 6/10 diabetic mice to achieve normoglycemia, although 1 subsequently rejected on day 185. None

of the untreated mice (n=3), nor the mice treated with DR80 alone (n=7), or anti-LFA-1 alone (n=3) achieved normoglycemia, suggesting it is the synergistic effect of these compounds that provides graft protection.

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ABC	Avidin-biotin complex
APC	Antigen presenting cells
B6	C57BL/6
CD	Cluster of differentiation
ConA	Concanavalin A
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DAB	3,3-diaminobenzidinetetrahydrochloride
DM	Diabetes mellitus
DR80	Direct Red 80
FACS	Florescence activated cell sorter
FICC	Fetal islet-like cell clusters
Foxp3	Forkhead box P3
HBSS	Hank's Balanced Salt Solution
HLA	Human leukocyte antigen
IBMIR	Instant blood mediated inflammatory reaction
ICAM-1	Intercellular adhesion molecule-1
Ig	Immunoglobulin
IL	Interleukin
LFA-1	Leukocyte function associated antigen-1
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
NK	Natural killer
NOD	Non-obese diabetic
NPI	Neonatal porcine islets
OCT	Optimum cutting temperature
PBS	Phosphate buffered saline
PD-1	Programmed death-1
PERV	Porcine endogenous retrovirus
SIM	Small inhibitory molecule
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha

CHAPTER 1

GENERAL INTRODUCTION

1.1 DIABETES MELLITUS

Diabetes mellitus (DM) constitutes a group of metabolic disorders that result in hyperglycemia, due to either the reduction in the secretion and/or action of insulin. Patients with this chronic condition suffer from abnormalities in fat, sugar, and protein metabolism, primarily leading to a state of hyperglycemia (1).

Worldwide, it is estimated that approximately 285 million people suffer from diabetes, a number that is expected to rise to 438 million by 2030 (2). Approximately 9 million Canadians are living with diabetes or pre-diabetes and the disease will contribute to the deaths of more than 41,500 Canadians per year. The life expectancy of a diabetic patient is estimated to be shortened by 10-15 years compared with the non-diabetic, and diabetic patients incur medical costs that are two to three times higher than the average Canadian. It is estimated that by 2020, diabetes will cost the Canadian healthcare system $16.9 billion per year (2). Debilitating secondary complications further contribute to the personal and economic burden of the disease. Direct patient costs for treating diabetes are estimated to range from $1,000 to $15,000 per year in Canada. All of these factors contribute to the decline in the quality of life of individuals with diabetes (2).

1.1.1 Classification of Diabetes

The majority of patients with DM fall into one of two major categories: type 1 and type 2 DM. Type 1 DM, also known as juvenile diabetes and insulin-dependent diabetes, is characterized by the absence of insulin, whereas type 2 DM is characterized by a combination of insulin resistance and inadequate compensatory insulin secretion. Type 2 DM is much more prevalent than type 1, however its clinical symptoms are generally less severe, and the use of exogenous insulin is not always necessary for survival. The risk of type 2 DM is increased with age, obesity, and sedentary lifestyle (1-3).

Type 1 DM can be further classified based on the serological evidence of autoimmunity. Type 1a is believed to result due to an immune-mediated destruction of beta cells within the islets of Langerhans. In contrast, type 1b is deemed to be idiopathic as there is no evidence of autoimmunity despite an absence of beta cells (3).

A third type of DM is gestational diabetes, a condition that occurs in 4% of pregnant women, and is characterized by insulin resistance (1). Other forms of the disease do exist, including genetic defects in beta cell function or insulin action and drug or chemical induced diabetes, however, these are very rare (3).

1.1.2 Anatomy and Physiology of the Pancreas

The pancreas is located in the retroperitoneum of the upper abdomen (4) and has both exocrine function, which aids in digestion, and endocrine function (Fig. 1-1). A normal adult pancreas contains approximately 1 million islet of Langerhans, the secretory component of the endocrine pancreas, which constitutes only 1-2% of the entire pancreatic mass. Although the islets are scattered throughout the pancreas, they are most concentrated in the body and tail. The main cells within the islets are the β cells, α cells, δ cells and pancreatic polypeptide (PP), which primarily secrete insulin, glucagon, somatostatin and pancreatic polypeptide, respectively (1).

Insulin is an anabolic hormone that is required for the achievement of metabolic homeostasis. It is secreted in response to high glucose levels and promotes the utilization and storage of fuels in the body (3). This is achieved via a number of mechanisms: *i)* suppression of hepatic glucose production (gluconeogenesis and glycogenolysis), lipolysis, and proteolysis ; *ii)* increasing the transport of glucose to adiopocytes and myocytes; and *iii)* stimulating glycogen synthesis (glycogenesis) (3). Insulin is synthesized in the beta cells of the pancreas as proinsulin. Cleavage of the molecule within the endoplasmic reticulum of the β cells leads to the removal of a connecting strand, or C-peptide, leaving a double-chain insulin molecule (5). Both insulin and C-peptide are released into the portal circulation at the same concentrations, however approximately 50% of secreted insulin is metabolized in the first pass through the liver. C-peptide also remains in the

circulation for a longer period of time, and is therefore often used as a marker for insulin secretion (5).

Insulin and glucagon have opposing effects, as glucagon stimulates the hydrolysis of glycogen by the liver, creating an increase in the blood glucose levels. Somatostatin functions to suppress the secretion of both insulin and glucagon, while the function of pancreatic polypeptide is currently unknown (1).

In type 1 DM, the destruction of the beta cells results in an absence of insulin production (1). As the body is unable to utilize the glucose, it must resort to the production of glucose through the breakdown of fats and proteins. In patients with type 1 diabetes, insulin is not present to antagonize the effects of glucagon and growth hormone, allowing these hormones continue to induce endogenous glucose production. Lipolysis is also unopposed, and there is an increase in the production of free fatty acids. This further increases ketogenesis and the inevitable result is ketoacidosis. The decrease in the pH of the body can interfere with enzymatic processes and eventually cause cardiovascular collapse. Protein metabolism is also uninhibited, resulting in muscle loss and cachexia (1).

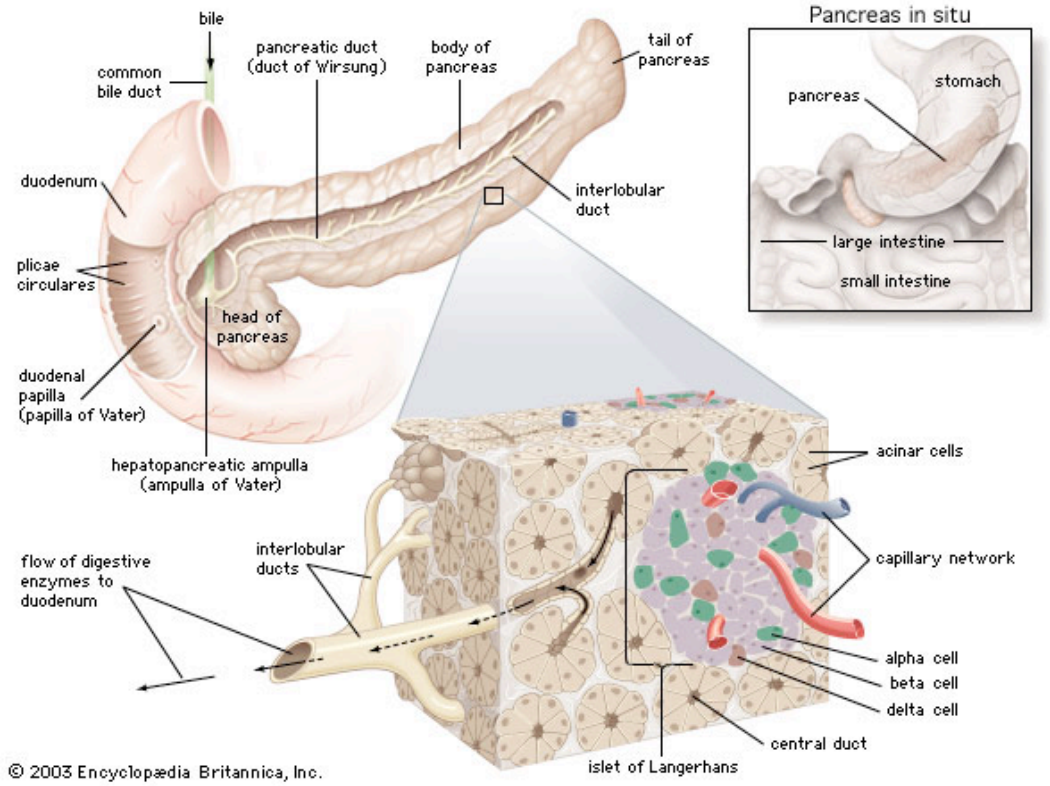

Figure 1-1. The pancreas and its associated organs.
Retrieved April 18, 2011 from Encyclopedia Britannica Online, http://www.britannica.com/EBchecked/media/101913/The-islets-of-Langerhans-are-responsible-for-the-endocrine-function (6)

1.1.3 Clinical Features and Complications

Clinically, diabetic patients may present with a variety of symptoms including polyuria, polydipsia, fatigue, polyphagia, weight loss and blurred vision (1). Long-term complications are a result of a persistent state of hyperglycemia, which induces damage to both large and small blood vessels, and can therefore be categorized as such. Complications involving small vessels include: *i)* retinopathy with potential vision loss; *ii)* nephropathy leading to renal failure; and *iii)* peripheral neuropathy, leading to decreased sensation. Large vessel disease is primarily mediated by the increase in

atherosclerosis seen in these patients, which eventually leads to myocardial infarctions, strokes, and aneurysms (1). Peripheral vascular disease is a combination of both small and large vessel disease, and leads to chronic foot ulcers and eventually amputations. Autonomic neuropathy may also occur, leading to genitourinary, gastrointestinal, and cardiovascular symptoms, as well as sexual dysfunction (1).

There are two major acute life-threatening complications of uncontrolled DM*: i)* diabetic ketoacidosis (DKA) and *ii)* non-ketotic hyperosmolar syndrome (1). DKA occurs primarily in type 1 DM patients, and is a state of hyperglycemia, ketosis and acidosis resulting from a deficient level of insulin and excessive secretion of counter-regulatory hormones. Severe electrolyte disturbances, shock, and even death can result. In non-ketotic hyperosmolar syndrome, type 2 DM patients also experience a relative insulin deficiency, however because there is a small amount of circulating insulin, lipolysis is inhibited, and therefore acidosis is prevented (1).

Hypoglycemia is the most common complication of insulin therapy for diabetes. In a normal state, a low blood glucose level elicits a physiologic response such as sweating, tremor, and palpitations, however in Type 1 DM, hypoglycemic unawareness can occur. This is particularly dangerous, as prolonged severe hypoglycemia can cause brain damage, coma, and even death (1).

1.1.4 Type I Diabetes Mellitus

Type 1a DM affects approximately 10% of diabetic patients (1,2), and selectively destroys the beta cells in the islets of Langerhans, while sparing the other cell types in the pancreas. These patients have a life-long dependence on exogenous insulin for survival, and in severe cases, can lead to glycemic lability and hypoglycemic unawareness (3). These "brittle" diabetics have large fluctuations in glycemic levels but lack the appropriate clinical symptoms such as sweating, tremors, tachycardia, and anxiety that signal a hypoglycemic episode (1,109). Hypoglycemic unawareness is particularly dangerous and can lead to seizures, coma, and even death. It is these patients who are currently the primary candidates to be recipients of islet transplants (109).

1.1.5 Etiology of Type 1 Diabetes

The current hypothesis regarding the development of type 1 DM is that an environmental trigger and an underlying genetic susceptibility initiates an autoimmune response targeted towards the β cells, resulting in their progressive destruction (1) (Fig. 1-2). The initial stages of disease development seem to be mediated by B cells which initially act as APC and then later secrete auto-antibodies. Cytotoxic T cells are able to kill β cells directly by inducing apoptosis through Fas-Fas ligand interaction, as well as the release of granzymes and perforin (7,8). T helper cells and macrophages secrete cytokines that direct the immune system towards a primarily Th1 response (7, 111).

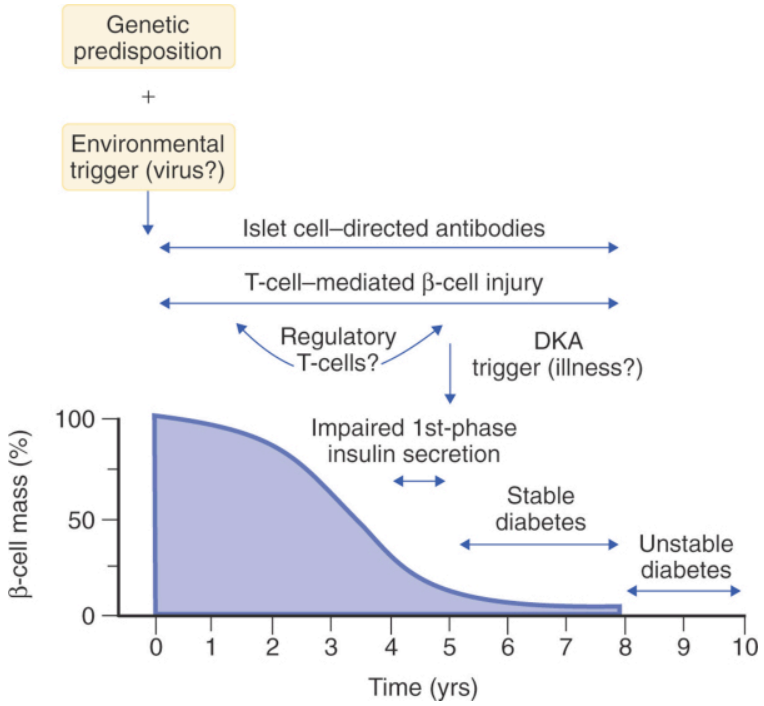

Figure 1-2: Sequence of events leading to the development of type 1 diabetes. Adapted from Goldman: Cecil medicine 23rd , Saunders Elsevier, Philadelphia, PA.

Genetic factors clearly play a role in the development of the disease. Although multiple genes have been identified as being linked with the development of type 1 DM (9), certain Human Leukocyte Antigen (HLA) genes on chromosome 6 are most often implicated. HLA genes encode the Major Histocompatibility Complex (MHC) molecules required for antigen presentation to T cells, and while some combinations of these genes result in protective effects against the development of type 1 DM, others tend to predispose (9). Monozygotic twin studies, however, prove that genetics are not the only factor involved in the development of the disease. If one twin is diagnosed with type 1 DM, the probability that their genetically identical

sibling will develop the disease is less than 40% (10). Therefore environmental factors, such as dietary components and viral infections, are hypothesized to contribute (10).

Dietary factors that have been linked to the development of type 1 DM include nitrosamine exposure, wheat proteins, and early exposure to cow's milk. While nitrates may have direct toxic effects on β cells via a mechanism similar to that of streptozotocin (126), the mechanisms underlying the link between type 1 DM and both cow's milk and wheat proteins are still uncertain (123,124,127,128).

Viral infections have been implicated due to a number of factors: *i)* new cases of type 1 DM show seasonal variation and clustering; *ii)* many new cases of type 1 DM occur after or during a viral infection (117); and *iii)* rubella infections *in utero* result in type 1 DM in 12-20% of individuals (118). Other viruses that are believed to contribute to the development of type 1 DM are coxsackie, mumps, and cytomegalovirus (CMV) (117). Possible mechanisms include the cross reactivity of the immune system's response to the virus with β cells (120), molecular mimicry of β cell antigens (119,121), and inducing β cells to express increased levels of HLA I and II (122).

1.1.6 Treatments for Type 1 Diabetes

The discovery of insulin in 1921 by Banting and Best (12) has made an immense impact on the treatment of patients with type 1 DM, transforming the disease from a fatal condition to a chronic one (1,110). Vigorous blood glucose monitoring and exogenous insulin administration is the crux of these

patients' treatment, as it leads to glycemic control and the reversal of the acute complications of diabetes (1,13). However, in order to decrease the onset and progression of secondary complications such as nephropathy, neuropathy, and retinopathy, strict glycemic control is necessary, and this is often difficult to achieve with these methods (13). In additional, intensive insulin therapy can result in an increase in the incidence of hypoglycemic episodes, which can ultimately lead to coma, seizures, and death (13). It is therefore necessary to develop a more physiological approach to the delivery of insulin for these patients.

1.2 ISLET TRANSPLANTATION

1.2.1 History of Islet Transplantation

The concept of islet transplantation actually predates the discovery of insulin itself. In 1892, Von Mering and Minkowski demonstrated that the pancreas was responsible for glucose control as dogs that underwent pancreatectomies subsequently developed hyperglycemia (14). They attempted the treatment of this hyperglycemia by subcutaneously transplanting portions of the pancreas, which led to temporary reduction in glycosuria (14). In 1893, Watson Williams attempted to transplant portions of a sheep pancreas subcutaneously to a young boy in diabetic ketoacidosis. Although there was temporary improvement in the boy's glycosuria, he ultimately died due to graft failure (15). Bensley in 1911 was the first to stain the islets of a guinea pig pancreas with a number of dyes, then picked them free from acinar tissue to study their morphology (131). The first attempt at

transplanting individual islets from the pancreas was in 1964 by Hellerstroem who used a free-hand microdissection technique (16). Moskalewski in 1965 first used a bacterial collagenase and mechanical disruption to digest a chopped guinea pig pancreas to improve the isolation and yield of islets (17). However, an adequate yield of islet for transplantation was first demonstrated by Lacy and Kostianovsky in 1967 (18). They obtained 300 islets from a rat pancreas, a modest yield, by cannulating and distending the pancreatic duct with Hanks Balanced Salt Solution (HBSS) prior to digestion with collagenase. They later used sucrose to provide a density gradient to isolate the islets from the contaminating exocrine and connective tissue (18). Lindall then substituted sucrose with ficoll, a polymer of sucrose, which improved islet purity while preserving function (19).

In vivo experimentation followed, and the first attempt at transplantation into a rodent was performed by Younoszai *et al.* in 1970 (20). They transplanted isolated rodent islets into a diabetic rat model, however they achieved only temporary normoglycemia (20). The first sustained reversal of hyperglycemia occurred two years later when Ballinger and Lacy transplanted 400-600 isolated rat islets into the peritoneal cavity or thigh muscle of rats that had become diabetic due to streptozotocin treatment (21). The first complete reversal of diabetes was demonstrated by Reckard and Barker, when they injected 800-1200 islets into the peritoneal cavity of hyperglycemic rats (22).

Larger animal pancreata proved to be more difficult to digest and often resulted in poor yields and viability. Unpurified pancreatic microfragments were investigated as sources of tissue during the 1970's (139-141). Horaguchi and Merrell introduced the concept of perfusion of the pancreas with collagenase via the pancreatic duct in order to cleave the islets from the collagen matrix (142). Pancreatic microfragments ultimately failed in clinical trials due to complications such as portal hypertension and disseminated intravascular coagulation (143). In 1989, Warnock *et al.* yielded purified human islets through the use of collagenase perfusion through the pancreatic duct, gentle mechanical dissociation, and ficoll-gradient purification (144), a process that is the basis of the islet isolation procedure to this day. Some refinements have been since developed, including the intraductal infusion of Liberase (collagenase blend), development of a semi-automatic dissociation chamber, and the utilization of a COBE 2991 cell processer for islet purification (112). These automated techniques increased the purity of the islets and reproducibility of the procedure. This in turn allowed for large-scale isolation, which was one of the first steps towards islet transplantation becoming a clinical reality (112).

The first clinical islet transplantation occurred in 1977 (25) and a number were performed over the following years. In 1989, Warnock *et al.* reported the outcome of islet-kidney allotransplantations, where approximately 5,000 IEQ/kg were infused into the portal vein. Although insulin requirements were reduced, patients ultimately lost graft function

(129). The development of a low-temperature tissue bank allowed for patients to receive a mix both fresh and frozen islets, increasing the number of total islets to greater than 10,000 islets per kilogram. This in turn allowed for sustained insulin independence in some patients (129). However, the majority of the subsequent islet transplantations were unable to sustain insulin independence for a minimum of one year. It was reported that only 8.2% of the 267 islet allografts that were performed in the 1990's maintained euglycemia for greater than 1 year (24). These first islet transplantations were generally performed in conjunction with a renal transplantation and with the immunosuppressive regimen including prednisone, azathioprine, and cyclosporine due to its success in solid organ transplantation. However, this regimen, in particular the glucocorticoid, proved to be toxic to the transplanted islets, which further limited its success (134,135).

In 2000, the Edmonton group reported in a landmark study that seven out of seven patients were able to achieve and maintain insulin independence for greater than one year post-transplantation, using the Edmonton Protocol. This was achievable for 2 reasons. The first is that a sufficient amount of islets were used for transplantation - a mean of 11,546 islet equivalents per kilogram of body mass were infused into the portal vein of the recipient. The second is that a less toxic glucocorticoid-free immunosuppression regimen was used, including sirolimus, tacrolimus, and anti-interleukin-2-receptor antibody therapy (daclizumab) (24). A five-year follow up of this study shows that the Edmonton Group has performed over 140 islet

transplantations in 85 patients (26). Despite the fact that 87% of patients maintained insulin independence for 1 year, only 9% of patients remained insulin-independent past this time point. However, glucose stability is improved, insulin requirements reduced, and hypoglycemic episodes are prevented (26). Further to this, C-peptide levels remained detectable in 80% of these patients, suggesting some level of graft function (26).

During the initial *in vivo* investigations of islet transplantation, the most common site of transplantation was the subcutaneous or intramuscular site, however a variety of other anatomic sites have been investigated in animal models with varied success (23,27). They include intraperitoneal, intraportal, instrasplenic, omental pouch, renal subcapsular, thymus, testicles, bone marrow, and even the ventricles of the brain (23). The most physiologic site, was determined to be intraportal injection, as under normal circumstances, the islets will secrete insulin into the portal vein (27). Clinically, intra-portal infusion of islets is relatively simple and can be performed using minimally invasive techniques with the patient returning home within days after the procedure (Fig. 1-3) (23,27). There are, however, disadvantages to this site: *i)* procedural complications such as bleeding, thrombosis, biliary leak, arteriovenous fistula, and a transient increase in liver enzymes, *ii)* increased intraportal pressure, *iii)* the islets are in direct contact with blood, therefore making them more susceptible to the instant blood mediated inflammatory reaction (IBMIR) and subsequent loss of tissue, *iv)* the islets are exposed to high concentrations of glucagon, diabetogenic

immunosuppressive drugs and their metabolites, as well as waste products from the gastro-intestinal tract, and *v)* monitoring the graft is difficult as biopsies are risky and difficult to perform (23,27).

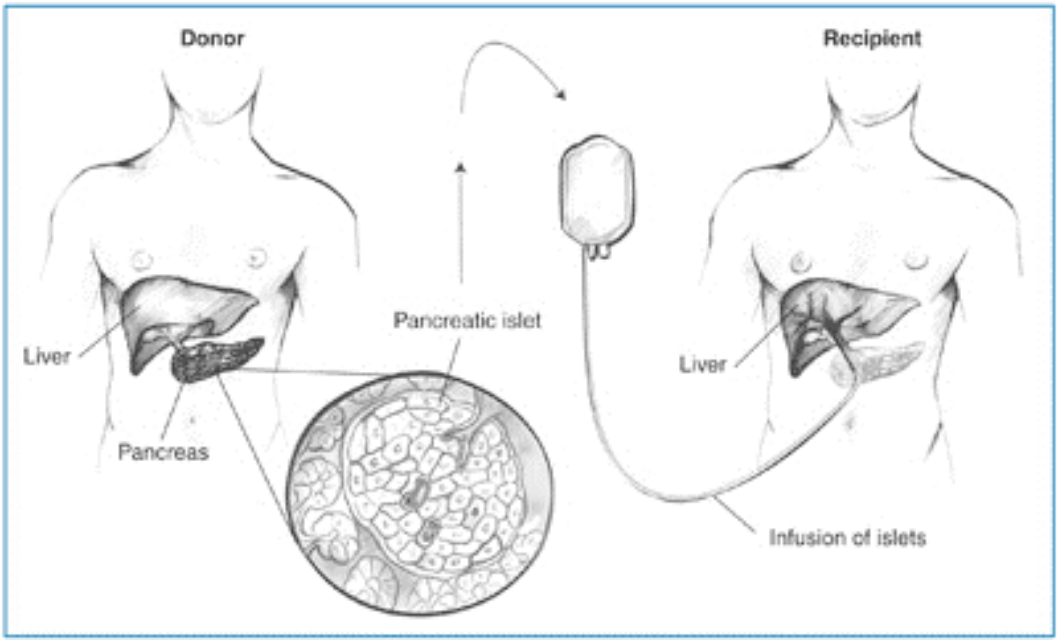

Figure 1-3: Human islet transplantation procedure. From National Diabetes Information Clearinghouse, *Pancreatic Islet Transplantation,* http://diabetes.niddk.nih.gov/dm/pubs/pancreaticislet/#2 (112)

1.2.2 Barriers to Islet Transplantation

There are 3 major barriers that are currently preventing the successful widespread implementation of islet transplantation for all type 1 DM patients: *i)* shortage of donor pancreatic tissue, *ii)* immune-mediated rejection of the graft and the required use of immunosuppressive drugs, and *iii)* the potential recurrence of autoimmunity.

1.2.2.1 Shortage of Donor Tissue

As with most clinical transplantation programs, the potential recipients far outnumber the donors. This is further compounded in islet transplantation, as multiple donor pancreases are required for a single transplant, and patients may require more than one transplant (24,26,28-32). There is likely early graft loss upon transplantation that may be due to a number of factors, including hepatic steatosis, ischemic injury, and the Instant Blood Mediated Inflammatory Reaction (IBMIR), which will be discussed further in section 1.3.3.1 (23,27). A potential solution to the problem of a shortage of donor tissue is the transplantation of insulin-producing cells from animal sources, or islet *xenotransplantation.* Multiple sources of xenogeneic islet tissue have been investigated for transplantation, including: *i)* bovine islets (33), *ii)* porcine islets (fetal, neonatal, and adult) (34-36) and *iii)* fish Brockmann bodies (37). Physiological and morphological similarities between human and pig islets make porcine tissue the most attractive xenogeneic source. Porcine insulin is structurally very similar to human insulin, differing only by one amino acid, and has been used clinically for the treatment of diabetes in humans for years (38). Pigs are also an attractive source as the breed rapidly, have large litter sizes, have a short gestation period, are relatively inexpensive, and can be housed in a pathogen-free environment (35,39). In addition, they can be genetically altered to create pigs that lack antigens readily recognized by the immune system,

therefore may limit the extent of immunosuppression required to prevent rejection (39).

1.2.2.2 Immune-Mediated Rejection of Transplanted Islet Grafts

The immune system provides a significant barrier to islet transplantation through a variety of mechanisms that will be discussed in detail in section 1.3.3. They include the activation of complement and coagulation cascades in the IBMIR, hyperacute rejection, and cell mediated rejection. To date, all recipients of organ transplants require continuous immunosuppression to prevent the rejection process. Chronic administration of immuno-suppressive drugs, however, results in significant toxicity and side effects, often limiting the patient population to which this treatment is appropriate (26). Some of the immunosuppressive medications currently being used clinically in islet transplantation are sirolimus, tacrolimus, and daclizumab and the reported side effects of these medications include mouth ulcers, ovarian cysts, diarrhea, acne, edema, anemia, and pneumonia (26). In order for the risk-benefit ratio to favor the use of clinical islet transplantation, only brittle diabetics are chosen as recipients (24,26,28,29,32,112).

1.2.2.3 Recurrence of Autoimmunity

Type 1 diabetes is an autoimmune disorder (1,7,8), and as such, the prevention of graft rejection is not relevant if the transplanted tissue is recognized by the preexisting autoimmune repertoire. If donor tissue shares epitopes with the target of the autoimmune cells and antibodies, they too

may be targeted and destroyed by the native disease process (63). This will be discussed in detail in section 1.3.3.4.

1.3 XENOTRANSPLANTATION OF PORCINE ISLETS

The ideal strategy in islet transplantation is to provide an unlimited source of islets while avoiding the need for immunosuppression. The transplantation of porcine tissue may provide a solution to the problem of donor tissue shortage.

1.3.1 Optimal Age of Porcine Islet Donor

Porcine islets are receiving support as a source of xenogeneic tissue for transplantation by many groups throughout the world, and are therefore being investigated extensively. Despite the enthusiasm for this technique, the optimal age of the porcine donor is still in debate. Three main age groups have been investigated, namely adult, neonatal and fetal. To date, successful large animal and non-human primate (NHP) islet xenotransplantation has only been achieved using neonatal and adult porcine islets (51-54), demonstrating the clinical potential of this tissue. However, there are several advantages and disadvantages to each group, which will be discussed below.

1.3.1.1Adult Porcine Islets

Adult pigs are a potential source of tissue, as the isolated islets function well both *in vitro* and *in vivo* immediately upon isolation, and the yield is substantial. Ricordi *et al.* demonstrated that approximately 255,000

islets could be isolated per adult pig pancreas, using a technique modified from the human islet isolation procedure. The final preparation was 85-90% pure and reversed hyperglycemia in nude mice (36). Since that time, adult porcine islets have been shown to reverse diabetes rapidly in NHP models (51, 52). Adult pig islets, however, do have their disadvantages. They are difficult to isolate and maintain in culture, are fragile, and are more susceptible to ischemic and hypoxic damage than neonatal porcine islets. In addition, they lack growth potential (40), which limits their ability to recover from any damage upon transplantation. Adult islets may also be relatively more immunogenic upon transplantation, which may only increase the need for immunosuppression (41). In order to be suitable for clinical transplantation, the pigs must be maintained in a pathogen-free environment until they are of an appropriate age for donation (39). This can be very costly and logistically very difficult, which can limit the applicability of adult pigs as islet donors.

1.3.1.2 Fetal Porcine Islets

The processing of fetal pig pancreas yield porcine fetal islet-like clusters (FICC), an immature group of cells that is capable of producing insulin. In 1988, a simplified procedure for the procurement of FICC was developed by Korsgren *et al.*, which is a simple procedure that does not require the ductal infusion of collagenase or ficoll gradient separation of islet cells that is seen in adult islet isolation (34). A media change every second

day purifies the cells, but the functional ability of the FICC is still poor due to their immaturity. They can be maintained in culture for up to 30 days, and are capable of proliferating, however, *in vivo* reversal of hyperglycemia in animals can take months (34). In addition, the yield of FICC per pancreas is low, and an estimated 100 porcine donors would be necessary to transplant one 70-kg patient (34).

1.3.1.3 Neonatal Porcine Islets

A functionally mature islet source with the isolation ease seen in fetal pig donors can be found in neonatal porcine donors. Korbutt *et al.* developed a protocol in 1996 for the isolation of neonatal porcine islets (NPI), which is easy to perform, with a consistent yield of 50,000 islets per pancreas. The preparation consists of 35% fully differentiated islets, and approximately 57% endocrine precursor cells (35). These precursor cells allow the islets to differentiate and divide in the post-transplantation period (35,39,42,43). NPI have been shown to reverse diabetes in both small and large animal models, including non-human primate (NHP) models, after a delay of up to 8 weeks due to their immaturity (35,44-49). They are also believed to be more resistant to hypoxic injury and less immunogenic than adult islets (41). In addition, neonatal pigs require fewer facilities and resources in order to house them compared with adult pigs.

There are two major disadvantages of using NPI as a source for transplantation. First, a yield of 50,000 NPI per pancreas translates to the

need for up to 70 donor piglets per patient, which is much greater than adult pig donors (35). Second, NPI express higher levels of antigens on their surface that can predispose the tissue to rejection upon transplantation. The most studied of these antigens is galactose α(1,3) galactose (Gal), which is the result of the enzyme α1,3-galactosyltransferase catalyzing the transfer of an α-galactosyl residue to a terminal β-galactose, and is present on all cell surface glycoproteins and glycolipids of all mammals except humans, apes, and Old World Monkeys (60). These animals develop antibodies due to exposure to the epitope through enteric bacteria and other pathogens. Although this may contribute to the xenorejection of porcine grafts, it is not likely the only xeno-antigen responsible for rejection (50).

1.3.2 Additional Barriers Limiting Application of Clinical Porcine Islet Transplantation

Two major obstacles currently limit the clinical application of porcine islet xenotransplantation. First, the safety of the donor tissue depends on the transmission of pathogenic infections/viruses, in particular, the transmission of porcine endogenous retrovirus (PERV). PERV is present in all pigs, as is a retrovirus encoded in their germline, and is therefore a potential source of xenosis. To date there has been no evidence of transmission of this virus to humans or non-human primates (51-54), however this virus is still a great concern – it has been shown that PERV from a porcine cell line can infect human kidney cells *in vitro* (55).

The second major obstacle to clinical applicability is to find a safe and effective way to protect the graft against the potent cellular response mounted against it. The requirement of immunosuppressive medications that have harmful side effects further limits the applicability of this treatment. Therefore safe methods of xenograft protection must be discovered in spite of the potent immune response.

1.3.3 Mechanisms of Porcine Islet Xenograft Rejection

After the transplantation of xenogeneic tissue, there are 3 major pathways of rejection that can destroy the graft in a rapid manner – the innate immune system, hyperacute rejection mediated by pre-formed natural antibodies and complement, and acute cell mediated rejection.

1.3.3.1 Instant Blood Mediated Inflammatory Reaction (IBMIR)

The innate immune system in the form of IBMIR is the major pathway of the first mechanism of graft loss. When islets are injected into the portal vein of the recipient, there are a number of factors that activate both the coagulation cascade and platelets (56). The intrinsic pathway of coagulation is activated due to the remnants of collagen in the graft, which is not normally in contact with blood. In addition, tissue factor (TF), which is expressed on both α and β cells, as well as on contaminating ductal structures, activates the extrinsic pathway of coagulation. Due to molecular incompatibilities between the xenogeneic tissue and the recipient, normal feedback mechanisms, such as porcine membrane TF pathway inhibitor,

which limit coagulation, do not occur (56). Platelets are also activated through the presence of thrombin, collagen, and von Willebrand factor bound collagen. These pathways lead to thrombus formation and subsequent ischemia and necrosis of the graft. The complement system is also activated and this is followed by infiltration of $CD11^+$ polymorphonuclear cells and macrophages, which lead to enzymatic digestion and phagocytosis of the islets, as well as the release of cytokines that can induce apoptosis (56,57). As a result, significant tissue damage occurs, and often the majority of the graft is destroyed within 24 hours. The damaged islets expel their insulin, and animals are at risk of becoming hypoglycemic. Although it has been demonstrated in primate models that insulin independence can be achieved even after such a significant loss of tissue, this is likely because a very large islet mass was transplanted initially. IBMIR is not limited to xenotransplantation, but the reaction is often more pronounced in this circumstance (56). Many strategies are being developed to attenuate this destructive reaction, including the addition of heparin in the islet preparation as seen in current clinical practice (58), the administration of low-molecular weight dextran sulphate, and various other compounds (56,59).

1.3.3.2 Hyperacute Rejection

Natural pre-formed antibodies can lead to a dramatic reaction and loss of the graft. In solid organ transplantation, discordant grafts are rejected due to antibodies to carbohydrate moieties, in particular, Gal. The aggressive

humoral response launched as a result of antibodies to Gal on solid organ xenografts can exhibit significant destruction, and IgG deposits are associated with the grafts by 12 hours post-transplantation. There is significant IgM and C3, C5, and C9 complement depositions within 2 days of transplantation.

Porcine islet grafts, however, seem to evade the hyperacute response that solid organ xenografts experience. The typical antibody and complement deposition that is seen in solid organ grafts is not observed when porcine islets are transplanted into non-human primates (52). In addition, the use of α1,3-galactosyltransferase gene-knockout (GT-KO) pigs as donors has not yet proved to reduce the post-transplant graft loss as compared to wild-type pigs (53). Only approximately 5% of adult pig islets express Gal on their surface, whereas approximately 20% of NPI express it (61). During both *in vitro* and *in vivo* maturation models of NPI, the expression of Gal is shown to reduce significantly as the precursor cells evolve into mature β-cells (61). Regardless, it has been demonstrated that NPI expressing and not expressing Gal can be susceptible to hyperacute rejection *in vitro*, suggesting that Gal is not the only xenoantigen responsible for this phenomenon (50). Islet xenografts are also mainly revascularized by recipient endothelial cells (62). It is for these reasons the natural pre-formed anti-Gal antibodies are not considered a major factor in the loss of islet graft tissue in the early post-transplant period (39,46,52).

1.3.3.3 Cell Mediated Rejection

Likely the most important mechanism of rejection of porcine xenografts is via T-cell mediated processes. T cell activation requires two distinct signals. The first signal is an antigen-specific signal that is provided by the interaction between a T cell receptor (TCR) and the foreign antigen. This antigen is presented in conjunction with an MHC molecule by an antigen-presenting cell (APC) to the TCR on the T cell. The second signal is also known as the co-stimulatory signal, and in the absence of an antigen-specific signal, is unable to activate T cells. Its role is to augment and amplify activation via decreasing the threshold of activation, increasing the expression of adhesion molecules, and preventing anergy. This process increases the production of cytokines as well as proliferation and differentiation of effector cells (114,115). In the absence of this co-stimulatory signal, T cells are believed to enter a state of anergy or unresponsiveness.

In mouse models, acute cellular rejection appears to be mediated predominately by $CD4^+$ T-cells, as CD8 knockout but not CD4 knock-out mice, reject their xenografts(116). In addition, due to the degree of phylogenic disparity between mice and the donor pig, the bias would be against direct recognition (63), or activation of T cells by the presentation of donor antigen by donor APC. The signals required for direct T cell activation may not occur because of incompatibilities of molecular interactions between the pig antigen presenting cells (APCs) and the mouse T cells (63,64). In genetically

modified mice, which are MHC II deficient and therefore lack an indirect response, the rejection of fetal porcine islet xenografts is delayed (64). The depletion of $CD4^+$ T cells further prolongs graft survival in these mice; however rejection is not completely prevented. This suggests that a direct response must occur by some mechanism upon T cell recovery in order for an immune response to occur (64). However, in NHP and human models, the direct presentation route may have increased significance compared to rodent models, as it has been demonstrated that both $CD4^+$ and $CD8^+$ human T-cells can respond to porcine APC (65). However studies to elucidate $CD8^+$ T cell's role *in vivo* in NHP have not been performed.

When taken together, the data suggests the likely mechanism of acute cellular rejection is the processing of porcine antigens by recipient APCs and presentation to recipient $CD4^+$ T cells, which can then activate the pathways necessary to destroy the xenograft. Macrophages, eosinophils, and T cells infiltrate the xenograft and reach a maximum infiltrate within 4-6 days (40). $CD8^+$ T cells may contribute to this rejection to a lesser extent, although the mechanism of their involvement is not clear. Their activation may be through either donor presentation (direct) or cross-presentation by the host APC (48). A major effecter mechanism may actually be through the activation of innate immune cells such as macrophages by antigen-specific $CD4^+$ T cells to kill the foreign cells by non-antigen specific mechanisms. Therefore as $CD4^+$ T cells are the most prominent cell type required for discordant islet xenograft rejection, treatments that specifically target this pathway without producing

toxic side effects will need to be found. In particular, therapies that induce tolerance to NPI xenografts may allow further clinical applicability of NPI xenotransplantation for the treatment of Type I DM.

1.3.3.4 Autoimmune Recognition

At present, the evidence suggests that the autoimmune repertoire of individuals with type 1 diabetes may be partially species-specific; therefore the xenogeneic tissue may escape the effects of the autoreactivity. However, the extent of this specificity is yet to be determined. If autoimmune cells and antibodies are able to target the xenogeneic tissue, it may be destroyed by the native disease process (63).

The non-obese diabetic (NOD) mouse was first reported in 1980 by Makino *et al.* after being developed at the Shionogi Research Laboratories in Japan (130). They have since been used as a model for autoimmune diabetes as these mice spontaneously begin to develop diabetes after 12 weeks, and 90% of female mice will be fully diabetic by 30 weeks (66). The onset of diabetes in male mice is delayed by several weeks, and the rates of disease are much lower, at only 20-30%. The mechanism of diabetes development is similar to that which is found in human type 1 DM patients, where autoreactive T cells infiltrate the islets and specifically attack the β cells (66).

Strategies that have been shown to be effective in chemically induced diabetic mice have not been effective in NOD mice (44,67). For example in our experience, the short-term administration of 2 monoclonal antibodies

(mAb), anti-LFA-1 and anti-CD154 mAb which are targeted against T cell activation, failed to promote survival of NPI xenografts in NOD mice despite being highly effective in B6 mice (44). Graft survival in the NOD mice required the administration of an additional monoclonal antibody against $CD4^+$ T cells, which led to xenograft survival in 9 of 12 mice, and long-term graft survival (>100 days) in 2 of these (44). Koulmanda *et al.* have also demonstrated that the depletion of $CD4^+$ T cells in NOD mice allowed for the prolonged survival of adult porcine islet xenografts (67). Interestingly, no further survival benefit was found when $CD8^+$ T cells were also depleted. However, further results have demonstrated that autoimmunity may not be the reason for the difficulty in inducing tolerance in NOD mice. When the NOD mice were treated with streptozotocin (STZ) prior to the onset of diabetes, autoimmunity was avoided, as subsequent islet isografts were not rejected as they are in spontaneously diabetic NOD mice. The prolongation of adult porcine islet survival by $CD4^+$ T cell depletion was virtually identical in both the spontaneously diabetic and STZ treated NOD mice, supporting the idea that recurrent autoimmunity does not substantially contribute to the rejection of islet xenografts (67). This conclusion is made cautiously though, as it may be $CD4^+$ T cell autoimmunity being prevented by treatment with the mAb (67). As well, the inability to achieve indefinite graft survival in all NOD mice with anti-CD4 mAb treatment may be a reflection of their ability to rapidly recover their $CD4^+$ T cell population (67, 138).

1.4 MONOCLONAL ANTIBODY (mAb) THERAPIES

1.4.1 Anti-LFA-1 mAb

Leukocyte associated antigen-1 (LFA-1) is a major adhesion molecule expressed on a variety of hematopoietic cells including macrophages, monocytes, granulocytes, natural killer (NK) cells, and with the greatest concentration on T and B cells. It is a β2 integrin with a heterodimer structure consisting of a CD11a α chain and a CD18 β chain (68-70). It binds to ICAM-1 and ICAM-2 on endothelial cells, ICAM-1 and ICAM-3 on antigen presenting cells (APC), and JAM-1 found on tight junctions of endothelial and epithelial cells (71,72). There are 4 major roles of this molecule that makes it an attractive molecule to target in order to prevent transplant rejection.

The first two of LFA-1's roles are involved in assisting leukocytes in their journey to the site of inflammation. ICAM-1 is expressed on the endothelial surface of the vessels near sites of inflammation, and as LFA-1 binds ICAM-1, it acts as a pro-migratory molecule, leading the leukocytes to these sites (72, 74). The binding of LFA-1 to ICAM-1 also assists in the actual extravasation of leukocytes out of the vessel. Leukocytes in blood and lymph vessels normally bind to L-selectin, which causes the cells to roll along the endothelial surface. When LFA-1 binds to ICAM-1, leukocyte rolling is arrested, which is necessary in order to move through the endothelial barrier to the tissues (Fig. 1-4) (72,73).

In order for T cells to be adequately activated, two major signals are necessary at the interface between an APC and the T cell – an antigen specific

signal and a co-stimulatory signal. LFA-1 plays a role in each of these signals (72). For the antigen-specific signal to cause sufficient activation, it must involve sustained periods of binding between the TCR and the APC – periods that cannot be achieved by simply engaging the TCR to the MHC molecule. Upon contact between the T cell and the APC, LFA-1 and ICAM-1 also bind. Significant rearranging of these molecules occurs in order to create a structure of adhesion molecules in a ring surrounding the engaged TCR in order to optimize its contact with the MHC molecule, leading to sufficient activation of the T cells (75).

With respect to LFA-1's involvement in co-stimulatory interactions, it appears that two major signals are sent upon contact with its ligands on APC. The first is an inside-out signal, which serves to increase the avidity of its own binding, while the second is an outside-in signal, which appears to be involved in intracellular signaling (76). LFA-1 binding with ICAM-1 also appears to enhance Ras activation, which is an important regulator of T cell development, homeostasis, and proliferation (72).

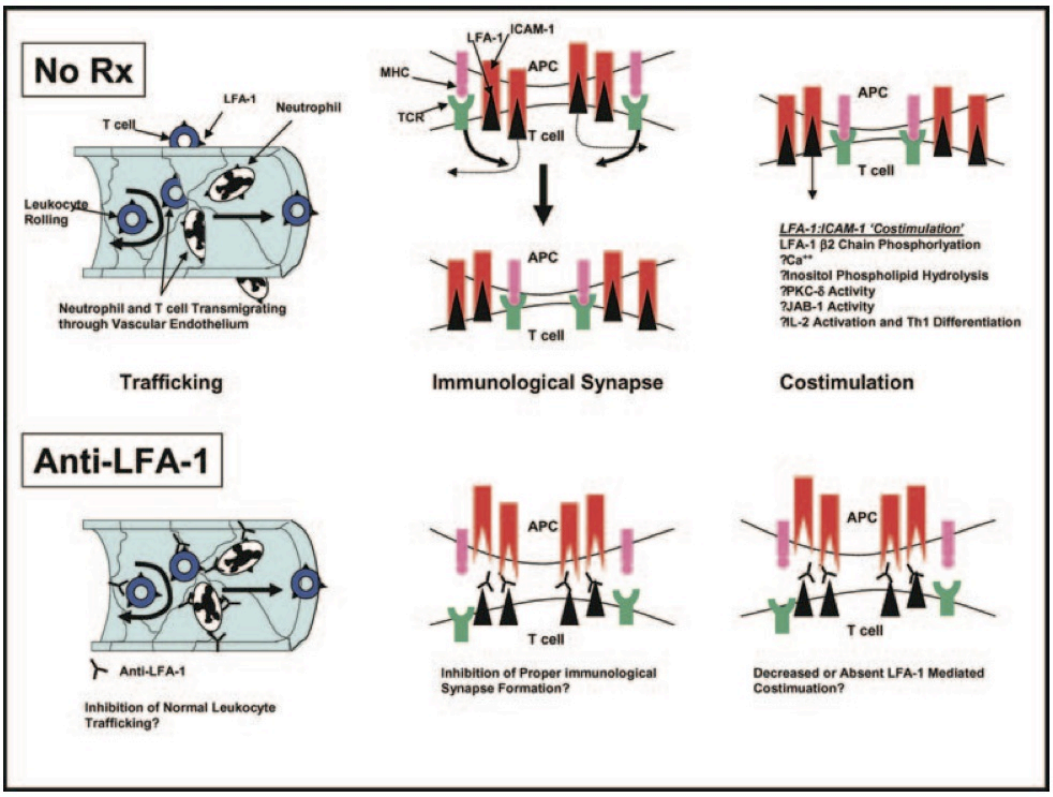

Figure 1-4: The actions of anti-LFA-1 mAb.
Nicolls, MR. & Gill, RG. 2006. LFA-1 (CD11a) as a therapeutic target. *Am. J. Transpl.* 6:27-36

The administration of this mAb has been shown to be effective in a number of allograft models, including cardiac, renal, and islet graft models (72,77,78). Clinically, the humanized form, Efalizumab, has been used to treat another autoimmune disorder, plaque psoriasis, with few acute side effects (79). There have been 2 trials to date in clinical islet allotransplantation that have reported its efficacy (80,81). In one trial, all 8 patients who received an islet transplant and were treated with an Efalizumab-based regimen achieved insulin independence; 4 of these patients achieved independence after a single islet transplant (80). In the second study, the 4 patients that

were treated with an Efalizumab based regimen achieved insulin independence after a single islet transplant (81). Efalizumab was ultimately removed from the market in 2009 due to 4 cases of progressive multifocal leukoencephalopathy (PML), however, none of the recipients in either of these studies demonstrated evidence of this disease (80,81). In fact, these 4 cases arose from over 40,000 patients who had been treated with the medication for greater than 4 years (81). Although the risk-benefit profile of this medication is unfavorable for the treatment of a relatively minor condition such as psoriasis, it is perhaps better than that of the traditional immunosuppressive agents currently being used in clinical transplantation. It is therefore still being investigated as a possible agent to prevent graft rejection.

With respect to xenotransplantation, the anti-LFA-1 mAb has been shown to be effective both *in vitro* and *in vivo* (48,82). In a concordant rat to mouse model, the administration of this mAb prevented islet graft rejection in 27 of 28 mice from up to 100 days (82). In a discordant NPI to mouse model, only 7/15 mice achieved normoglycemia with the short-term administration of anti-LFA-1 mAb, and only 6/15 maintained long-term graft survival (48). Anti-LFA-1 mAb has also been shown to improve the function of adult porcine islets in mice when added to a CTLA4Ig and anti-CD154 mAb regimen (83). Collectively, these studies showed that the anti-LFA-1 mAb can be efficacious in preventing islet allograft rejection, and is very promising in

the prevention of xenograft rejection, however monotherapy with this medication is not sufficient.

1.4.2 Anti-CD154 mAb

CD154, which is a member of the TNF family and is found on activated T and B cells, activated platelets (85), and many other inflammatory cells (86,87). CD40 is a trans-membrane glycoprotein and a member of the TNF-receptor superfamily. It is expressed on a number of cells including activated T cells, all APC (B cells, dendritic cells, macrophages), hematopoetic progenitor cells, and endothelial cells (84). The binding of these molecules sends co-stimulatory signals to both the T cell and the APC. Cytotoxic and helper T cells are primed and proliferate due to the up-regulation of co-stimulatory signals and IL-2 receptors, as well as the release of a number of cytokines (84,91).

Binding to CD40 by CD154 is critical in the maturation process of the APC, as it promotes the expression of other co-stimulatory molecules (ie: CD80 and CD86) and adhesion molecules such as ICAM-1 (84). B cell maturation and differentiation into plasma cells is dependent on these co-stimulatory signals, which promotes their clonal expansion, and the ability of the cells to class switch antibody isotypes (84). It also promotes antigen presentation by the APC (84), and increases the production of inflammatory cytokines and chemokines such as TNF-α, IL-12, IL-8 (88,89).

The disease, Hyper IgM Syndrome, clearly demonstrates the importance of the CD40-CD154 interaction. In this disorder, the CD154 gene is defective, and the result is a normal to high level of IgM, with low levels of other antibody isotypes (IgG, IgE, IgA) due to the inability to class switch (90). This, combined with defective priming of T cells, leaves the patient with a severe immune deficiency (90).

In NHP models of islet allotransplantation, the administration of the humanized form of anti-CD154 mAb (hu5c8) has demonstrated significant efficacy in preventing rejection (92,93). In three of three baboons that received islet allografts and were treated with short-term anti-CD154 mAb, delayed rejection of their grafts was observed. This rejection was reversed by the re-administration of the mAb (92). In rhesus macaques, six of six recipients of islet allografts and anti-CD154 mAb induction therapy plus monthly maintenance therapy achieved and maintained insulin independence for >100 days, with no evidence of rejection in 5 of the 6 animals (93). Thus in allotransplantation, the benefits of the anti-CD154 mAb are clear.

In NPI xenograft models, the administration of anti-CD154 mAb (MR-1) as a monotherapy has yielded only modest results, with approximately 40% of diabetic mice achieving normoglycemia (48). However, combination therapy of anti-LFA-1 and anti-CD154 mAbs has improved survival of porcine islet xenografts. In mice transplanted with NPI, short- term treatment with

both anti-LFA-1 and anti-CD154 mAbs allowed 100% of the mice to demonstrate long-term survival of the grafts (>100 days) with only 10% rejecting prior to 300 days post-transplant (45). These results are substantial and clearly demonstrate that simultaneous interference of adhesion and costimulatory pathways can lead to islet xenograft tolerance. Pre-clinical models with the transplantation of either NPI or adult porcine islets have demonstrated that the humanized form of anti-CD154 mAb (H106, ABI793) is very effective when combined to existing regimens, such as basiliximab (anti-CD25 mAb), sirolimus, and FTY720 or belatacept (CTLA4-Ig) (46,51,52).

The side effect of the humanized form of anti-CD154 mAb is that it has been shown to increase the incidence of thrombo-embolic events in both human and non-human primates (94). Activated platelets express CD154, which would likely be bound by the mAb. In platelet rich plasma, the anti-CD154 mAb did not seem to induce aggregation, nor did it affect maximal aggregation, however it dose-dependently inhibited the de-aggregation of activated platelets. It is proposed that the CD154 expression on physiological, or possibly pathophysiological, platelets can sustain a pro-aggregatory effect of the Ab by a mechanism involving the Fc domain. (96)

When treated with the mAb to prevent renal allograft rejection, 4 of 9 rhesus macaques developed significant thromboembolisms. Administration of heparin reduced the number of events to 2 in 10, and ongoing treatment

with heparin reduced this even further. Phase-1 clinical trials have also reported similar numbers, halting the clinical investigation. The administration of heparin concomitantly with the mAb did decrease this incidence, however it remains above the acceptable limit for clinical use (94). Non-human primate models also suggest that the administration of aspirin during the treatment with anti-CD154 mAb therapy could greatly reduce the incidence of these events (95).

1.5 SMALL INHIBITORY MOLECULES

1.5.1 Introduction to Small Inhibitory Molecules

Therapeutic mAb have been enthusiastically researched in recent years, as they are attractive in their abilities to bind to a specific target and are stable in human serum. There are disadvantages to their use, however: *i)* poor oral bioavailability, therefore limits in their clinical usage; *ii)* difficult and expensive to manufacture; *iii)* not cell-permeable, therefore antagonism of intracellular protein-to-protein interactions (PPI) is not possible (97). For these reasons, the development of small inhibitory molecules has been met with great enthusiasm.

Small inhibitory molecules are molecules that are able to block protein to protein interactions by binding to small but high affinity areas called “hot spots”. They are very specific and as such, their administration may avoid some of the undesirable side effects of currently used monoclonal antibodies (97-100).

The development of small inhibitory molecules is difficult, as there are few naturally occurring small molecules that bind at protein interfaces. In addition, the usual size of a protein-to-protein interface is relatively large, and lacks an obvious site of binding for small molecules on X-ray crystallography (97). By identifying the hot spot within the interaction, scientists are able to identify molecules with a complementary structural conformation that have the ability to bind at these sites (97-100).

1.5.2 Suramin

Suramin sodium is a hexasodium salt and a derivative of naphthalenetrisulfonic acid, with a molecular weight of 1429.17g/mol (101-105). Clinically, it is being used as a prophylactic treatment for parasitic infections, including trypanosomiasis (African Sleeping Sickness) and helminthiasis (104, 105). It has also been investigated as an anti-neoplastic drug, and has shown some efficacy in the treatment of prostate cancer (106).

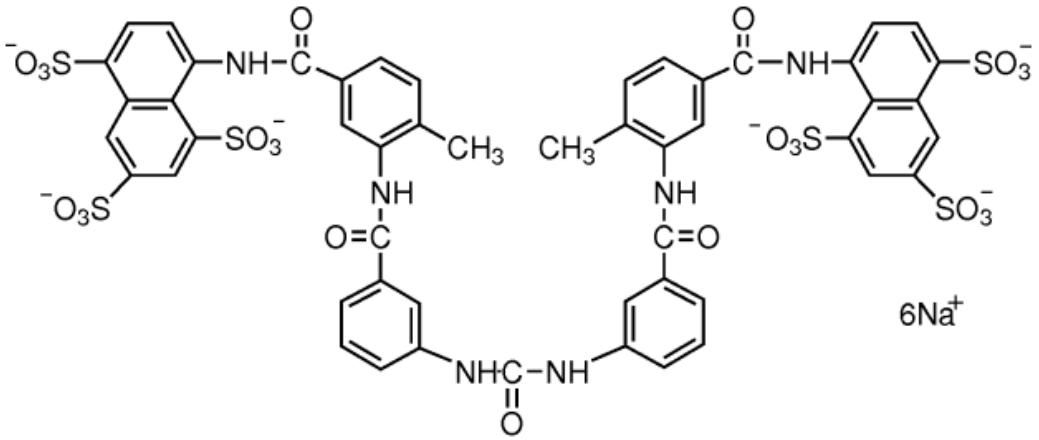

Figure 1-5: The chemical structure of suramin. From Sigma-Aldrich website, Retrieved April 21, 2011, http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=en&N4=S2671|SIGMA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC (107)

Suramin has also been demonstrated to cause a degree of immunosuppression and have anti-proliferative effects on lymphoid cell lines. It prevents binding of several growth factors to their cell surface receptors, including epidermal growth factor, tumor-derived growth factor β, and platelet-derived growth factor (101-103). It also appears to block the binding of IL-2 to its cell surface receptor, a cytokine that is critical to the T cell response of the immune system (105).

In vivo testing of suramin demonstrates that it induces thymic atrophy and profound splenic lymphocyte depletion in mice, with little effect on other organs. It is also known to inhibit coagulation by altering levels of heparin and dermatan sulfate (101-103,105).

Margolles-Clark *et al.* identified suramin as a molecule capable of blocking the CD40-CD154 interaction *in vitro* (108). In cell-free binding studies, suramin blocked both the human and mouse CD40-CD154 interaction in a concentration dependent manner, with a half-maximal inhibitory concentration (IC50) of 18.9-20.9μg/mL and 6.43μg/mL respectively (98,99). When cell culture media is also added, which takes into account non-specific protein binding but better replicates the *in vivo* environment, the IC50 does increase to 89.12-98.2μg/mL (98,99). When compared with the human anti-CD154 mAb, suramin's IC50 is more than 170,000 times greater, indicated less activity (98,99). Suramin did, however, show considerable selectively to this particular interaction. B cell

proliferation was also inhibited *in vitro* by suramin, with an IC50 comparable to its binding assay results (124.8-137.5μg/mL), and without any significant cytotoxicity (98,99). This group also showed that suramin could decrease the release of pro-inflammatory cytokines that are known to be detrimental to islets after transplantation, such as IL-6, IL-8, and IFN-y (108).

Due to its known effects on the CD40-CD154 interaction, this group used an iterative activity screening and structural similarity search procedure to identify other compounds that could also potentially block this interaction. The result was a series of organic dyes including Direct Red 80 (DR80) (99,100).

1.5.3 Direct Red 80

Direct Red 80 (DR80), also referred to as picrosirius red, is an organic azol dye generally used in the textile industry. It has a molecular weight of 1373.07g/mol, and a chemical formula of 2-Naphthalenesulfonicacid,7,7'-(carbonyldiimino)bis[4-hydroxy-3-[2-sulfo-4-[(4-sulfophenyl)azo]phenyl]azo, hexasodiumsalt (107).

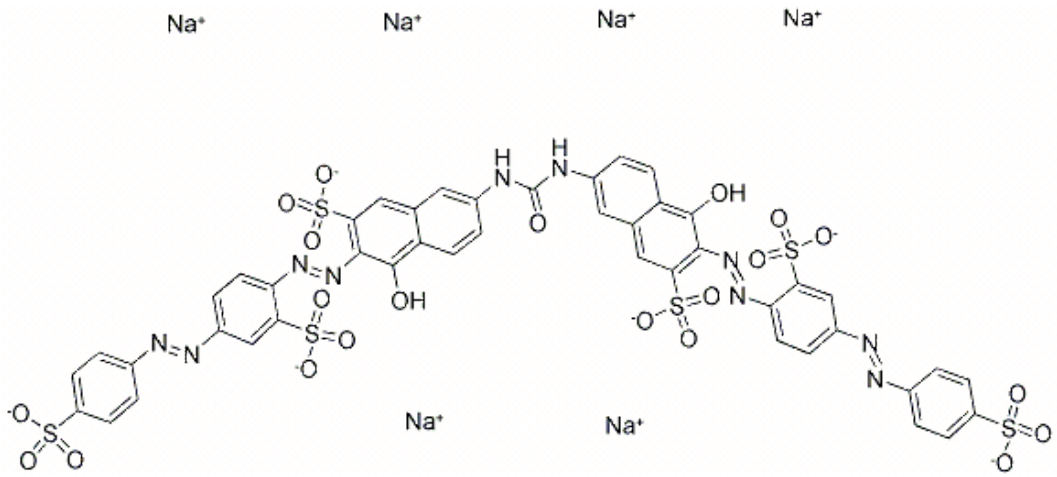

Figure 1-6: The chemical structure of DR80. From Sigma-Aldrich website, Retrieved April 21, 2011, http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=en&N4=S2671|SIGMA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC (107)

DR80 was identified as a small inhibitory molecule that is capable of blocking the CD40-CD154 interaction in a dose dependent manner (99,100). In cell free binding assays of CD40-CD154, the IC50 of DR80 was found to be 2.47-4.39μg/mL for human, and 8.10μg/mL for mouse molecules (99,100). When cell growth media was added, the activity was reduced about 10-fold, in that the IC50 for the human molecules was 24.17μg/mL. B cell proliferation assays were also performed, using human CD19+ B cells and stimulation with rhIL-4. DR80 was found to inhibit this activation with an IC50 of 47.23μg/mL (99). Again, the human anti-CD154 mAb was found to be much more active, with an IC50 44,000 times smaller. DR80, like suramin, did not display any evidence of cytotoxicity, and was found to be specific for the CD154 molecule (99,100).

The *in vitro* evidence suggests that these two compounds, suramin and DR80 are promising therapeutic agents that effectively block the CD40-CD154 interaction in a dose dependent manner. However, to date, there have been no *in vivo* studies performed to assess their efficacy in preventing islet graft rejection.

1.6 OBJECTIVES AND OUTLINE

The blockade of the LFA-1/ICAM-1 and CD40/CD154 interactions is an attractive strategy in the prevention of rejection of xenografted NPI in mice (45,46,51). The use of an anti-CD154 mAb has been shown to induce thromboembolic events in humans and NHP, therefore more specific therapies need to be developed (94). Promise lies in the development of small inhibitory molecules such as organic dyes that bind to CD154 in relatively small "hot spots" and may not induce these same side effects (97-100,108).

In Chapter 2, both *in vitro* and *in vivo* studies examine the anti-proliferative effects of suramin and DR80. Direct toxicity assays were performed to assess if the compounds will directly harm NPI. *In vitro* proliferation assays were performed via mixed lymphocyte reaction and mitogen stimulation assays, in order to assess the inhibition of lymphocyte proliferation by the same compounds. Finally, *in vivo* experiments were performed. C57BL/6 mice were treated with streptozotocin to render them hyperglycemic, then transplanted with NPI and injected with the SIM alone, and in combination with α-LFA-1 mAb. At 150 days post-transplant, mice

with protected grafts underwent a survival nephrectomy of the graft-bearing kidney to demonstrate a return to a hyperglycemic state. Mice that rejected their grafts were euthanized at this time. The morphology of the grafted tissue was examined by immuno-histochemistry to assess for insulin positive cells and infiltration of immune cells. The mouse spleens were also collected and analyzed using flow cytometry to characterize the phenotype of the immune cells present.

The studies described were designed in order to address the barriers of clinical islet transplantation in the treatment of diabetes. By using porcine islets, the issue of a shortage of donor tissue is addressed, however rejection of this tissue is still a problem. Current therapies that block an important signal in transplant rejection are harmful, inducing thrombo-embolic events, therefore we attempt to find a specific small inhibitory molecule that can inhibit this interaction, without inducing harmful side effects.

We hypothesize that the SIM suramin and DR80 will inhibit the activation and proliferation of T cells, and will prevent the rejection of NPI xenografts in mice. The overall goal of these studies is to investigate strategies to prevent rejection of islet xenotransplants, in order to facilitate the development of this as a viable treatment of diabetes. Therefore, the objectives of this thesis project are: *1)* determine if the SIM suramin and DR80 are toxic to NPI; *2)* determine if suramin and DR80 can inhibit T cell proliferation *in vitro;* and *3)* determine if suramin and DR80 can prevent the rejection of NPI xenografts in a diabetic mouse model.

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CHAPTER 2

DR80 INHIBITS T CELL ACTIVATION AND PREVENTS THE REJECTION OF NPI XENOGRAFTS IN MICE IN COMBINATION WITH ANTI-LFA-1 mAb

2.1 INTRODUCTION

Type 1 diabetes mellitus is a chronic metabolic disorder characterized by the autoimmune destruction of the insulin producing β cells within the islets of Langerhans in the pancreas. (1-3). Traditionally, these patients are treated with intensive insulin therapy in order to maintain normoglycemia (1,4,5). Islet transplantation is an alternative therapy in the treatment of type 1 diabetes mellitus, which offers a more physiologic delivery of insulin, and may prevent the debilitating secondary complications of the disease (6-10). Its use however, is limited to a very select group of patients considered to have "brittle" diabetes (6,7,10). This is in part due to the shortage of human donor pancreatic tissue and the continuous use of harmful immunosuppressive drugs to prevent rejection of the islet transplant. Xenogeneic tissue, in particular neonatal porcine islets (NPI), is being considered as an alternative source of islets for clinical transplantation. They are relatively easy to isolate, purify, and maintain in culture, and have the ability to proliferate and differentiate (11-13,15). They have been shown to reverse diabetes in small and large animal models (16-20), including the preclinical monkey model (21).

The other barrier to clinical islet transplantation is the requirement for the continuous use of immunosuppressive drugs that have harmful side effects (6-10). The administration of anti-LFA-1 monoclonal antibody (mAb) in combination with an anti-CD154 mAb has been found to be very effective in preventing the rejection of NPI in B6 mice (17). In both human and non-human primates, however, the anti-CD154 mAb has been shown to increase the incidence of thromboembolic events, effectively halting preclinical studies (22,23). An alternative molecule to this mAb that has the ability to target the CD40-CD154 interaction specifically, without inducing thromboembolic events, is being sought.

Small inhibitory molecules (SIM) have been a focus of research because of their ability to block protein-to-protein interactions in a very specific manner by binding to small but high affinity areas on molecules called "hot spots" (24). Suramin is a compound that was initially identified as capable of interfering with the CD40-CD154 interaction *in vitro*. An iterative search to find molecules with similar structures and binding capabilities revealed a number of organic dyes. Direct Red 80 (DR80), an organic textile dye, was found to be one of the most active compounds *in vitro*, capable of blocking the CD40-CD154 interaction in a dose-dependent manner in micromolar concentrations, with minimal cytotoxicity. To date, no *in vivo* studies involving suramin or DR80 and their use in islet transplantation have been performed (24,25). We hypothesize that suramin and DR80 will inhibit T cell proliferation *in vitro* and will prevent the rejection of NPI xenografts in

mice. The objectives of this study were to determine the toxicity of suramin and DR80 to NPI and to confirm their inhibition of the co-stimulatory interaction CD40-CD154 *in vitro.* We also sought to determine whether these molecules could replace the anti-CD154 mAb in combination with anti-LFA-1 mAb and prevent rejection of NPI xenografts in mice.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Two to 3 day old neonatal pigs of either sex (1.2-2.2 kg, University of Alberta farm, Alberta, Canada) were used as islet donors. Six to eight week old male C57BL/6 (B6, H-2^b, Jackson Laboratory, Bar Harbor, ME, USA) mice were used as transplant recipients, with B6 *rag*$^{-/-}$ (B6.129S7-Rag1^{tm1mom}/J, H-2^b) as control recipients. The mice were rendered chemically diabetic by a single intraperitoneal (ip) injection of streptozotocin of 200mg/kg and 185mg/kg body weight respectively (Sigma, St Louis, MO, USA) 3-5 days prior to transplantation. Blood glucose levels were measured twice weekly using a One Touch Ultra glucose meter (Lifescan Inc., Milpitas, CA, USA). All diabetic mice had two consecutive fasting blood glucose levels of ≥17 mmol/L prior to transplantation. All animals were fed standard laboratory food and cared for according to the guidelines established by the Animal Welfare Committee at the University of Alberta and the Canadian Council on Animal Care.

2.2.2 Isolation of NPI

Neonatal pigs were placed under anesthesia with isoflurane, and a laparotomy and exsanguination was performed. The pancreas and spleen were dissected free and placed in Hank's Balanced Salt Solution (HBSS) with 0.25% (w/v) bovine serum albumin (BSA, fraction V; Sigma). The pancreas tissue was chopped into 1 mm fragments with scissors and digested with Type XI collagenase (1mg/ml; Sigma). The tissue was then filtered through a 500μm nylon mesh and cultured for 7 days in Ham's F10 medium, containing 10mmol/L D-glucose, 50 μmol/L isobutylmethylxanthine (ICN, Biomedicals, Montreal, QC, Canada), 0.5% BSA, 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, and 95% air. The NPI media was changed on post-isolation days 1, 3, and 5 (11). On post-isolation day 7, the islets were collected and aliquots counted as islet equivalents (IEQ), using 150μm as the standard for 1 IEQ (11).

2.2.3 Isolation of Pig Splenocytes

Two to 3 day old pigs were placed under anesthesia with isoflurane and a laparotomy and exsanguination was performed. The spleen was dissected free and placed in HBSS as above. The tissue was then transferred to a petri- dish containing sterile saline and chopped into small segments using a sterile scissors. The fragments were then squished using the rough edges of frosted microscope slides to free the splenocytes from the surrounding capsule and connective tissue. The cells were separated from

the tissue and placed in red blood cell lysing buffer to rid the sample of any red blood cells. The cells were then frozen in 10% dimethyl sulfoxide (DMSO) and 90% FBS at -80°C (17).

2.2.4 Isolation of Mouse Splenocytes

Naïve male B6 and BALB-c mice are placed under anesthesia with isoflurane and cervical dislocation is performed. A laparotomy and splenectomy is performed, and the tissue is placed in sterile saline to be processed as described above for pig splenocytes (17).

2.2.5 NPI Toxicity Assays

Suramin (Sigma), DR80 (Sirius Red; Sigma), mycophenolate mofetil (MMF) (Roche), Tacrolimus (Astellas Pharma USA), Sirolimus (Wyeth-Ayerst Canada Inc.), and Cycolsporine A (Sigma) were prepared in Ham's F10 and filtered to achieve sterility. Doubling dilutions were performed to obtain the required concentrations. Aliquots of 100 islet equivalents in 1mL of Ham's F10 that had been cultured for 7 days were incubated for 24 hours in an additional 1mL of varying concentrations, ranging from 0 to 50nM, of either suramin, DR80 or the conventional immunosuppressive drugs in a 24 well plate. After 24 hours of incubation, the islets were collected, and washed with HBSS. Their viability was assessed with trypan blue.

2.2.6 Mitogen Stimulation and Mixed Lymphocyte Reaction Assays

The assays were prepared as per our lab's previously published protocols (17, 30). Naïve B6 mice (n=3) were immunized by transplanting

2,000 NPI under the kidney capsule. After 21 days, the mouse was euthanized; its spleen was collected and the murine splenocytes were isolated as described above. Five hundred thousand mouse splenocytes are incubated in a 96 well plate with the following conditions: 1) Alone; 2) With ConA (mitogen stimulation); 3) With 300,000 porcine splenocytes (of the same pig that the mouse was initially immunized with) (mixed lymphocyte reaction – MLR); 4) With 300,000 BALB/c splenocytes to act as an allograft control. Both the porcine and BALB/c splenocytes had been irradiated using 25 Gray (2500 rad) gamma radiation in order to render them non-proliferative. The cells were incubated for 1 to 6 days, at which time they are pulsed with [^{3}H] (thymidine) and then incubated for an additional 18-24 hours. At this time they are harvested onto glass microfiber filters (Wallac, Turku, Finland) and counts per minute (cpm) are detected using the Wallac MicroBeta Trilux luminescence counter (PerkinElmer, Waltham, MA) (17, 30). The days of maximum proliferation were determined based on the results of these experiments, and were found to be days 1 and 4 for the mitogen stimulation and the MLR, respectively.

The experiments were then repeated on four separate occasions, now incubating the murine splenocytes with varying concentrations of Suramin and DR80, from 0μg/mL to 1mg/mL, each concentration in quadruplicate. [^{3}H] was added to the plates with Con A after 1 day and to the plates with porcine splenocytes and islets on Day 4. They were harvested after 24 hours and radiation counts per minute were assessed using a beta counter.

2.2.7 Transplantation of NPI

After 7 days of culture, the NPI were collected and counted using the method previously described by Korbutt *et al.* (11) to determine the number of islet equivalents (IEQ) in each preparation. One IEQ is equal to 150μm. Aliquots of 2,000 IEQ were prepared and transplanted under the left kidney capsule of streptozotocin-induced diabetic B6 mice. The aliquots were aspirated into polyethylene tubing (PE-50) and pelleted via centrifugation, then placed within the subcapsular space of the left kidney using a micromanipulator syringe. The capsule is then cauterized to seal the puncture.

Graft function in recipient mice is indicated by normoglycemia, defined as blood glucose levels <10mmol/L. Graft rejection is defined as the first of 3 consecutive readings of >10mmol/L. To ensure that normoglycemia was a function of the graft, all normoglycemic mice underwent a survival nephrectomy of the graft-bearing kidney at 150 days, to demonstrate the return to a hyperglycemic state.

2.2.8 In Vivo Therapies

Transplant recipient mice were randomly allocated to different treatment groups to receive either no treatment, or one the following therapies intra-peritoneal (ip): 1) Suramin alone, 250μg on days 0, 1, then Mondays and Fridays for 4 weeks; 2) Suramin and anti-LFA-1 mAb (200μg on days 0, 1, 7, 14); 3) DR80 alone (250μg on days 0, 1, then Mondays and

Fridays for 4 weeks); 5) DR80 and anti-LFA-1; 6) anti-LFA-1 mAb and anti-CD154 mAb (MR-1; hamster IgG1; Bioexpress, West Lebanon, NH, USA; 250μg on days -1, 1, 4, 7, 11, 14, 18, 21, 25, 28); 7) anti-LFA-1 mAb alone.

In addition B6 rag-/- mice were transplanted with NPI in order to demonstrate their function. Mice were followed for 150 days by monitoring their blood glucose levels twice weekly.

2.2.9 Oral Glucose Tolerance Tests

Mice that achieved stable normoglycemia for ≥2 weeks underwent an oral glucose tolerance test (OGTT) (17). The mice were fasted for 12-15 hours and then administered 50% dextrose orally at a dose of 3g/kg. Blood glucose levels were measured at times 0, 15, 30, 60, 90, and 120 minutes.

2.2.10 Immunohistochemical Analysis of Graft Sections

At the end of the study, the pancreas and graft bearing kidneys were harvested and fixed with 10% buffered formalin solution, then embedded in paraffin. Sections (5μm thick) were stained to determine the presence of insulin secreting cells. The sections were quenched with 10% hydrogen peroxide in methanol, blocked with 20% normal goat serum (Cedarlane, Burlington, ON, CA) then incubated for 30 minutes with guinea pig anti-insulin antibody (1:1000 dilution, Dako Laboratories, Mississauga, ON, CA). The sections were then incubated for 30 minutes with biotinylated goat anti-guinea pig IgG secondary antibody (1:200 dilution, Vector Laboratories, Burlingam, CA, USA). Avidin-biotin complex/horseradish peroxidase

(ABC/HP, Vector Laboratories) and 3,3-diaminobenzidinetetrahydrochloride (DAB, BioGenex, San Ramon, CA, USA) complex to produce the brown color seen in the slides. They were then counterstained with Harris' hematoxylin and eosin (16, 17).

The subpopulations of immune cells within the graft were characterized from cryopreserved sections. Once the islet graft was removed, it was immersed in optimum cutting temperature (OCT) solution and snap frozen at -80°C. Sections were immersed in acetone at -20°C for 4 minutes. The sections are blocked with 2% FBS in PBS for 20 minutes and then are incubated with an avidin/biotin blocking kit for 10 minutes each to block endogenous biotin or biotin-binding proteins (Vector Laboratories). The primary Abs of rat anti-mouse CD4 (1:500, BD Pharmingen), rat anti-mouse CD8 (1:200, BD Pharmingen), rat anti-mouse CD11b (1:300, BD Pharmingen), and rat anti-mouse CD19 (1:200, BD Pharmingen) are applied to the sections and incubated for 1 hour. The secondary Ab, biotin anti-rat IgG (1:200, BD Pharmingen), is then added for 30 minutes. As in the paraffin embedded sections, avidin-biotin complex/horseradish peroxidase (ABC/HP, Vector Laboratories) and 3,3-diaminobenzidinetetrahydrochloride (DAB, BioGenex, San Ramon, CA, USA) complex were added to form the brown color in the slides. They were then counterstained with Harris' hematoxylin only (17).

2.2.11 Flow Cytometric Analysis of Immune Cell Phenotype

Spleens from all B6 mice and 3 naïve mice were collected at the end of the study. Splenocytes were isolated and aliquots of 1 x 10^6 spleen cells were

incubated for 30 minutes at 4°C with fluorescent conjugated antibodies (1:100 dilutions; eBioscience, San Diego, CA) specific for lymphocyte cellular markers. Analysis was performed using a LSR II flow cytometer, with a gate on the live spleen cells (BD Biosciences) (17).

2.2.12 Detection of Mouse Anti-Porcine IgG Antibody Levels in Mouse Serum

At the time of euthanasia, a blood sample is obtained from the transplanted mice through cardiac puncture, and the sera is isolated by centrifuging the blood at 8000rpm for 15 minutes and collecting the topmost later. Porcine spleen cells (1×10^6) obtained from the same pig donors were incubated with diluted mouse serum (1:128) for 1 hour at 37°C, 5% $C0_2$, and 95% air. Spleen cells were then washed with PBS and incubated with goat anti-mouse IgG fluorescein isothiocyanate (FITC) labeled antibody (1:200 dilution, Southern Biotechnology Associates, Inc., Birmingham, AL, USA) for 1 hour at 4°C. Using the LSR II flow cytometer, the viable spleen cells were gated and the percentage of cells bound to antibody was detected. Naïve B6 mice, pig cells incubated without serum or secondary Ab, and pig cells incubated with secondary Ab but in the absence of mouse serum all served as controls (17).

2.2.13 Statistical Analysis

Statistical analysis for all data was performed using Graphpad Prism 5. Toxicity assays were analyzed using a Student's t-test; *in vitro* proliferation assays were analyzed with both 2-way ANOVA and a 4-parameter non-linear

regression. Flow cytometric and OGTT data was analyzed using a 2-way ANOVA and Tukey's method for multiple comparisons for post-hoc analysis. P values of less than or equal to 0.05 were considered statistically significant.

2.3 RESULTS

2.3.1 Suramin and DR80 are not significantly toxic to NPI compared to conventional immunosuppressive drugs

NPI were incubated in various concentrations of suramin and DR80 as well as conventional immunosuppressive drugs including Mycophenolate Mofetil (MMF), Sirolimus, Tacrolimus, and Cyclosporine A. While MMF and Sirolimus in particular appeared to be quite toxic to the NPI, especially at higher doses, neither suramin nor DR80 exhibited any significant toxicity (Table 2-1).

Anti-Rejection Therapy	**n**	**Percent live cells at various concentrations of anti-rejection therapies (nM)**										
		0	5	10	15	20	25	30	35	40	45	50
Suramin	3	100	100	88	91	94	100	100	97	94	100	100
DR80	3	100	100	100	100	100	98	100	100	91	97	94
MMF	3	100	98	94	97	100	88	97	81	94	80	70
Sirolimus	3	100	94	95	100	94	91	88	84	92	73	65
Tacrolimus	3	100	98	97	94	78	94	94	94	91	93	91
Cyclosporine A	3	100	97	88	91	94	100	94	91	88	88	88

Table 2-1: **Viability of cells within NPI after incubation in suramin, DR80, and various immunosuppressive medications.** NPI were cultured in Ham's F10 media and exposed to each compound for 24 hours. Each condition was performed in triplicate. Live cells were detected by incubation and examination with trypan blue.

2.3.2 Suramin and DR80 inhibit T-cell proliferation in vitro

To assess if suramin or DR80 were capable of inhibiting T cell activation *in vitro,* mitogen stimulation and mixed lymphocyte reaction (MLR) assays were performed. In order to demonstrate the day of maximal proliferation, and therefore the day in which inhibition may be most pronounced, preliminary studies were performed in the absence of the small inhibitory molecules. Maximal proliferation occurred on days 1 and 4 for mitogen and MLR assays respectively. (Fig. 2-1)

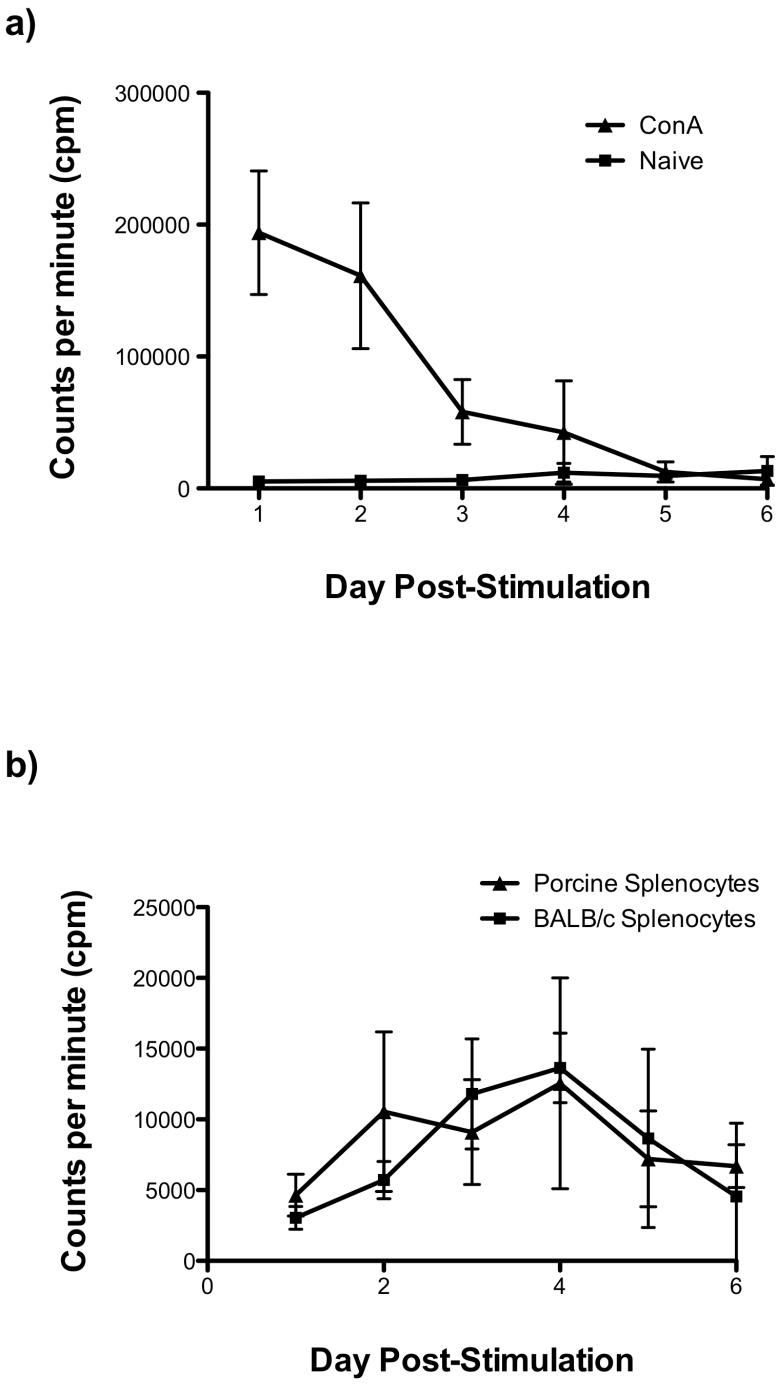

Figure 2-1: Proliferation of murine splenocytes after stimulation with a) ConA and b) porcine or BALB/c splenocytes. The days of maximal proliferation for each were determined to be Day 1 and Day 4 respectively.

The studies were repeated in the presence of suramin or DR80. Both molecules exhibited significant inhibition of T cell proliferation *in vitro* in a dose dependent manner (Fig. 2-2, 2-3, 2-4, 2-5).

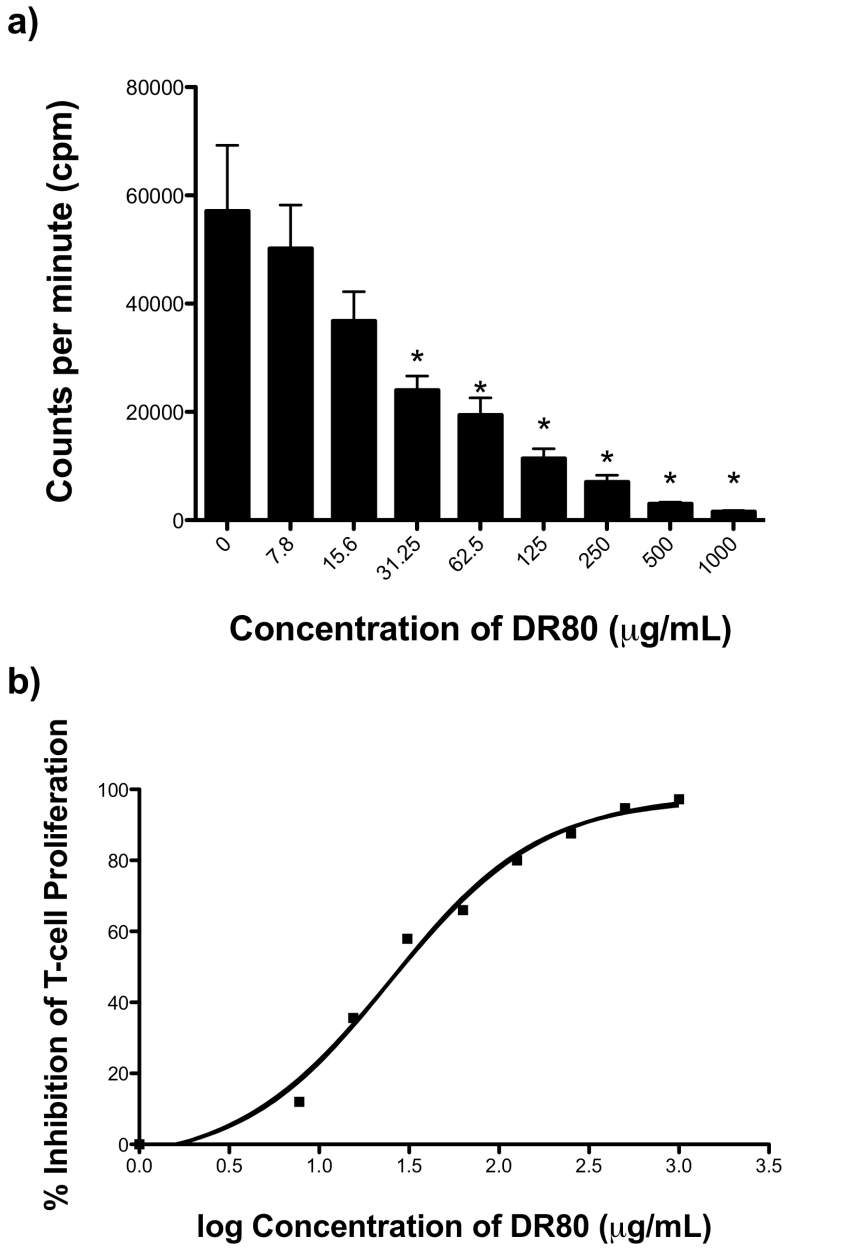

Figure 2-2: Proliferation of murine splenocytes after stimulation with ConA and incubation with DR80 (a). Percent inhibition of proliferation with DR80 (b) is also shown. The IC50 is 24.86μg/mL. *p<0.05, when compared to proliferation of splenocytes in the absence of DR80.

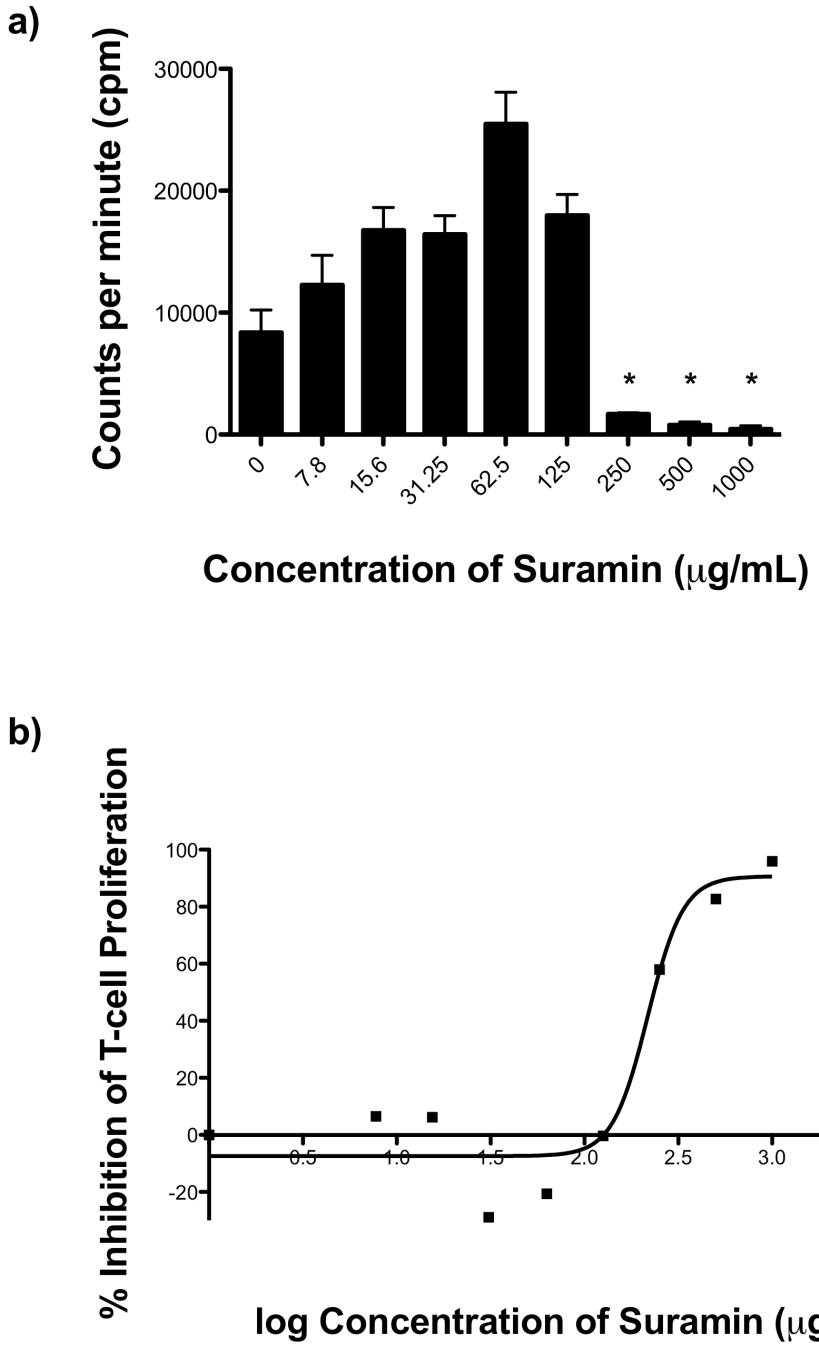

Figure 2-3: Proliferation of murine splenocytes after stimulation with ConA and incubation with suramin (a). Percent inhibition of proliferation with suramin (b) is also shown. The IC50 is 217.7μg/mL. *$p<0.05$, when compared to proliferation of splenocytes in the absence of suramin.

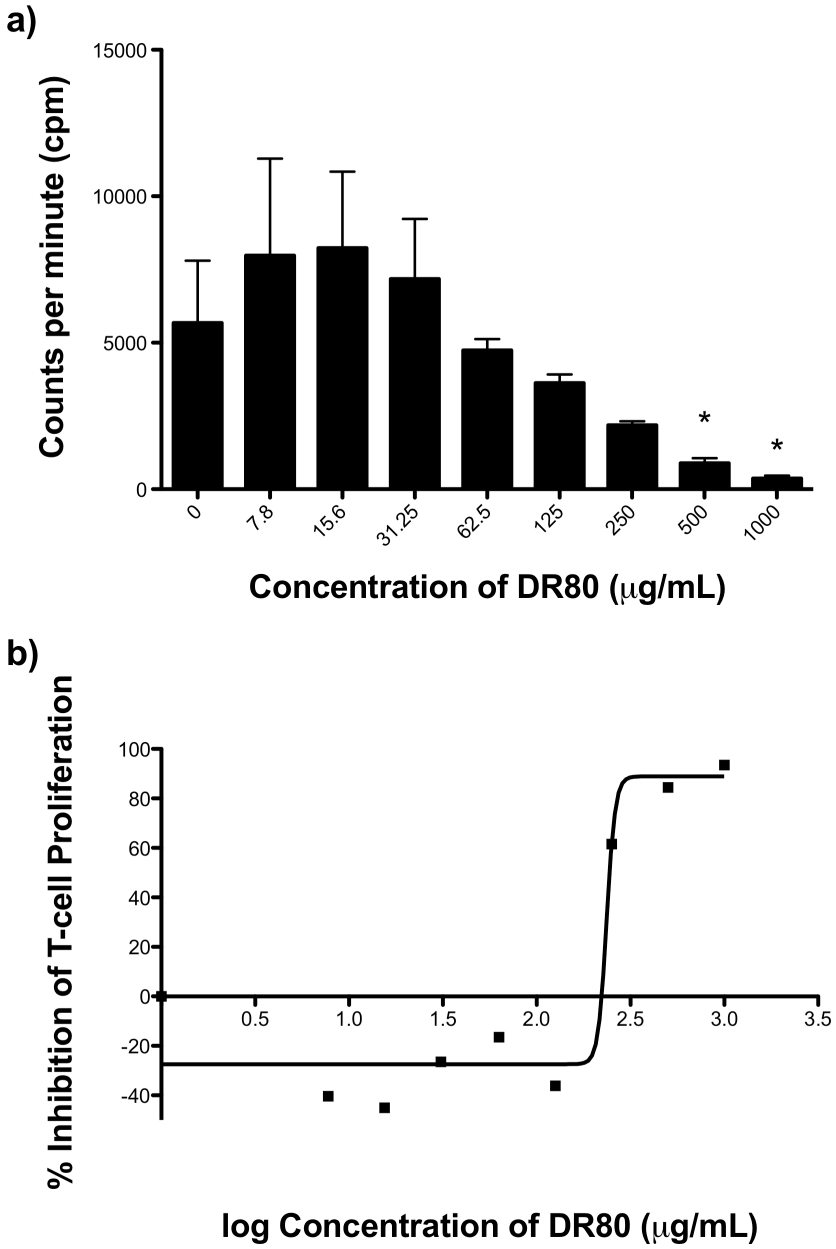

Figure 2-4: Proliferation of murine splenocytes after stimulation with porcine splenocytes and incubation with DR80 (a). Percent inhibition of proliferation with DR80 is also shown (b). The IC50 is 236.4μg/mL.*$p<0.05$, when compared to proliferation of splenocytes in the absence of DR80.

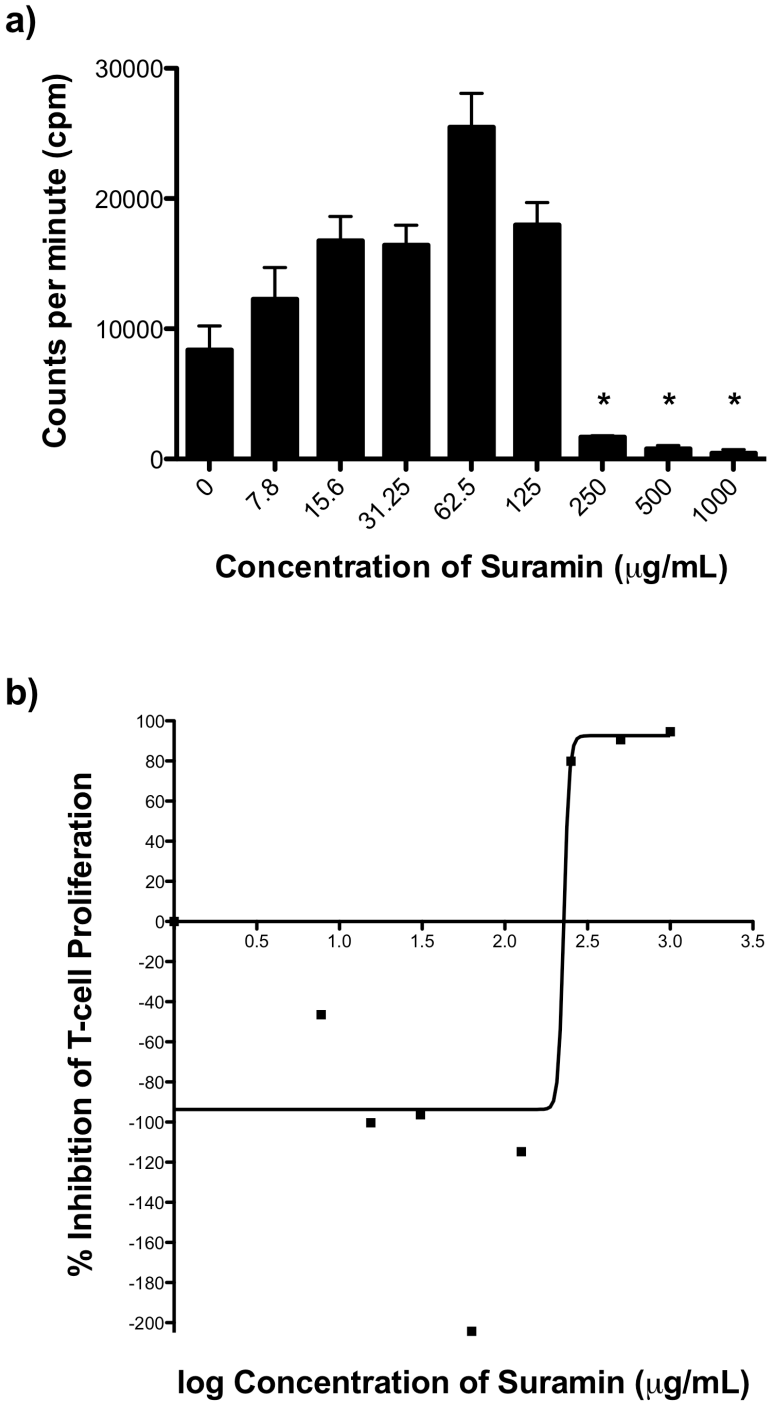

Figure 2-5: Proliferation of murine splenocytes after stimulation with porcine splenocytes and incubation with suramin (a). Percent inhibition of proliferation with suramin is also shown (b). The IC50 is 227.6μg/mL. *$p<0.05$, when compared to proliferation of splenocytes in the absence of suramin.

In comparison with Margolles-Clark *et al.* findings, our results were similar. In those assays, B cell proliferation was stimulated in a non-antigen specific manner through the use of rhIL-4, and reported an IC50 for suramin and DR80 of 124.8-137.5μg/mL and 47.23μg/mL respectively (98-100). These methods are most comparable to our non-antigen specific mitogen stimulation assays, in which the IC50 for suramin and DR80 were found to be 217.7μg/mL and 24.86μg/mL respectively. Our antigen specific assays resulted in higher IC50 for both suramin and DR80, at 227.6μg/mL and 236.4μg/mL respectively. This is the first time these compounds have been evaluated in this manner. Viability studies reveal that neither of these compounds exhibited significant cytotoxicity to mouse splenocytes at any of the examined doses (Fig. 2-6).

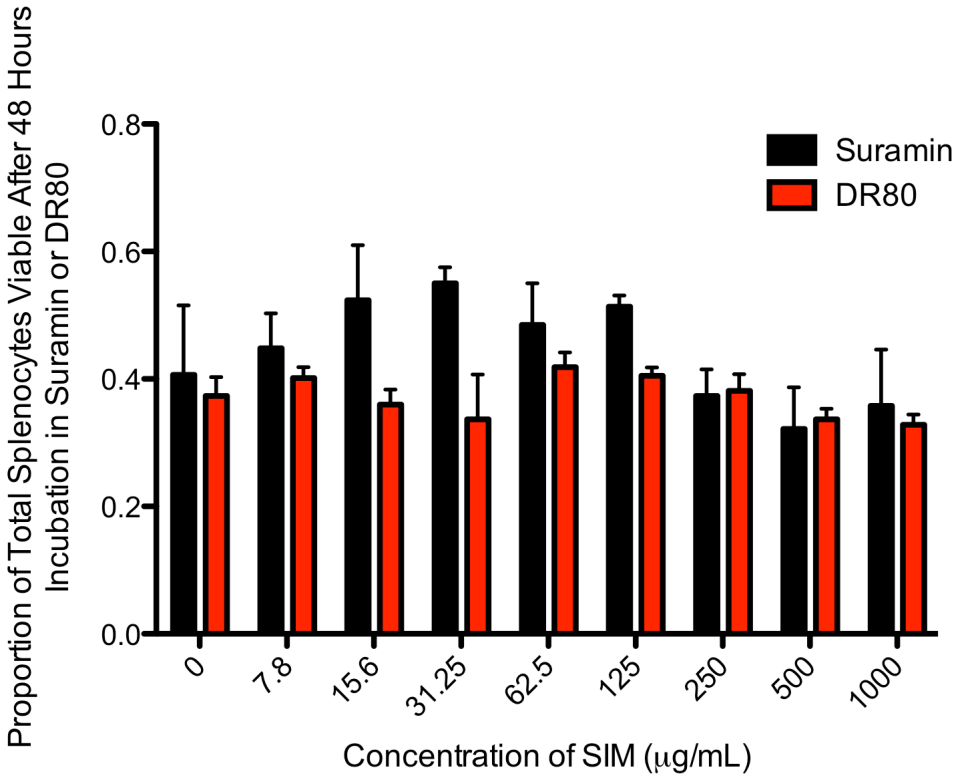

Figure 2-6: Viability of mouse splenocytes after 48 hours incubation in varying concentrations of suramin and DR80. No significant differences were found compared with viability in the absence of these molecules.

2.3.3 DR80 in combination with anti-LFA-1 prevents the rejection of NPI xenografts in mice

Suramin and DR80 were administered to B6 mice that had received NPI xenografts in order to determine if these molecules are capable of preventing xenograft rejection (Table 2-2). None of the mice that were left untreated after transplantation (n=4) achieved normoglycemia. When treated with Suramin (n=6), DR80 (n=10), or anti-LFA-1 mAb (n=4) as monotherapy, the mice still remained diabetic, suggesting that any one of these molecules alone is not sufficient to prevent rejection. When suramin was combined with anti-LFA-1 mAb only 1/5 mice (20%) achieved

normoglycemia and 3/5 required euthanasia by day 50 due to poor health. DR80 in combination with anti-LFA-1 mAb in contrast led to 6/10 mice (60%) achieving normoglycemia. One mouse subsequently rejected the graft at day 185 post-transplant. This was also in contrast to the mice treated with α-LFA-1 mAb and α-CD154 mAb in combination (n=5). One of these mice achieved normoglycemia, while the remainder did not. This was surprising due to the much more positive results that had previously been found with this treatment (17), however, it is possibly due to poor quality of islets as the two B6 rag -/- mice that received NPI from the same donor pig also did not achieve normoglycemia. One mouse treated with suramin and anti-LFA-1 mAb also received the islets of questionable quality. It is therefore possible that these mice may have reached normoglycemia if better quality islets were transplanted.

Treatment	N	Graft Survival (days)	Number of Recipients that Achieved Normoglycemia
No Treatment (NPI)	4	0, 0, 0, 0	0
α-LFA-1 mAb	4	0, 0, 0, 0	0
Suramin	6	0, 0, 0, 0, 0, 0	0
DR80	10	0, 0, …	0
α-LFA-1 mAb + α-CD154 mAb	5	0, 0, 0, 0, 167	1
Suramin + α-LFA-1 mAb	5	0, 0, 164, 0, 0	1
DR80 + α-LFA-1 mAb	10	185, 185, 228, 185, 145, 145, 0, 0, 0, 0	6

Table 2-2: Graft Survival in B6 mice transplanted with NPI and treated with mAb and SIM. Mice received no treatment, anti-LFA-1 mAb alone, suramin alone, DR80 alone, or anti-LFA-1 mAb in combination with either anti-CD154 mAb, suramin or DR80. Blood glucose levels were monitored twice weekly. Graft survival of 0 days indicates the mouse did not achieve normoglycemia.

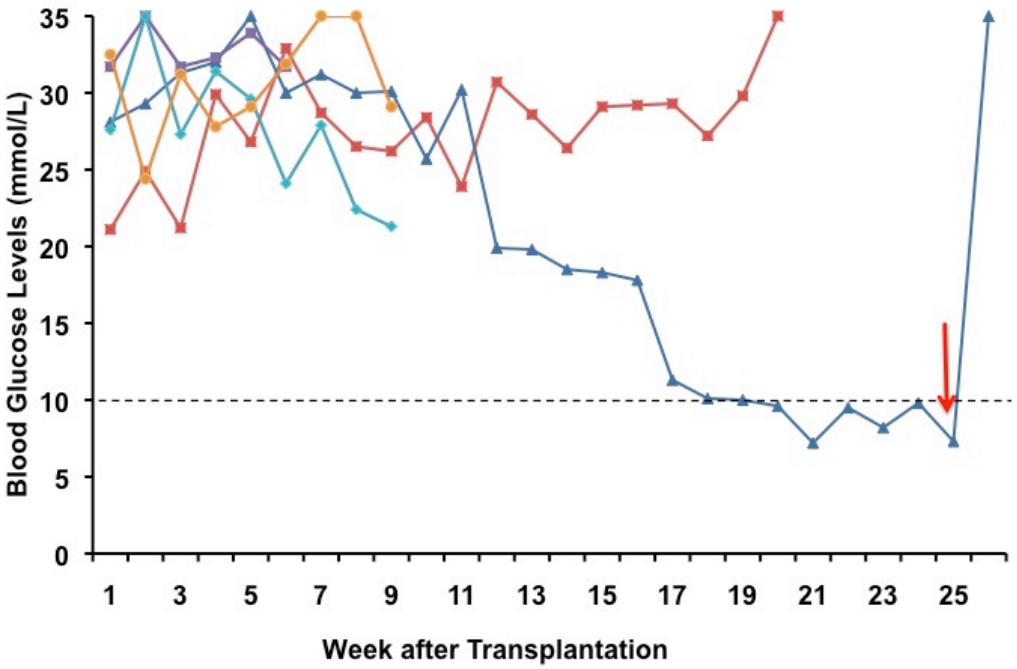

Figure 2-7: Blood glucose levels of mice treated with Suramin and anti-LFA-1 mAb. Only 1 mouse achieved normoglycemia at 16 weeks post-transplantation. The red arrow indicates a survival nephrectomy with a subsequent return to hyperglycemia.

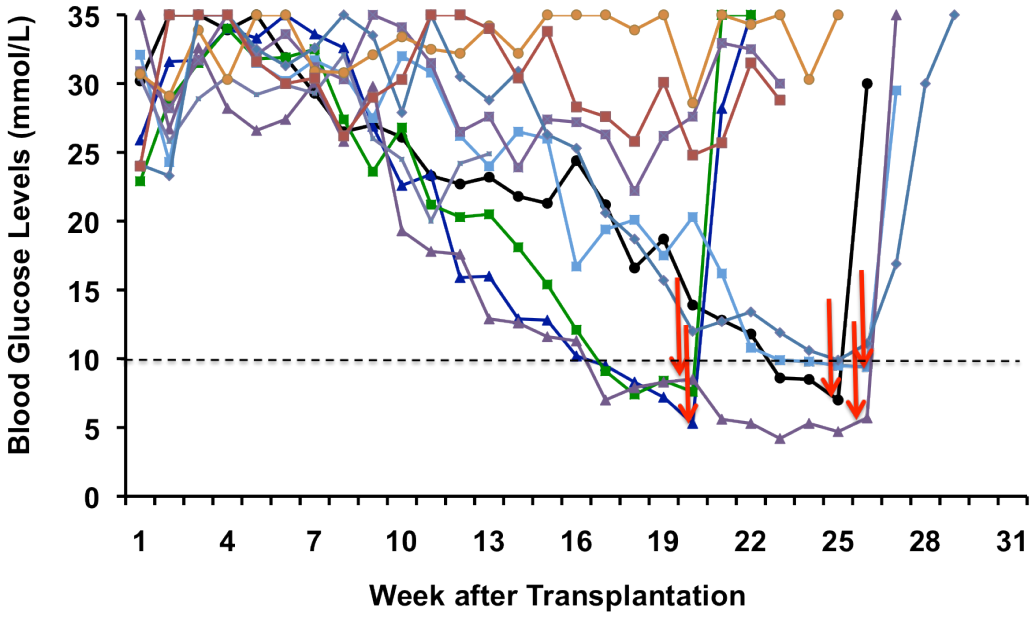

Figure 2-8: Blood glucose levels of mice treated with DR80 and anti-LFA-1 mAb. Red arrows indicated survival nephrectomies, with a subsequent return to hyperglycemia. One mouse rejected at 185 days post-transplantation.

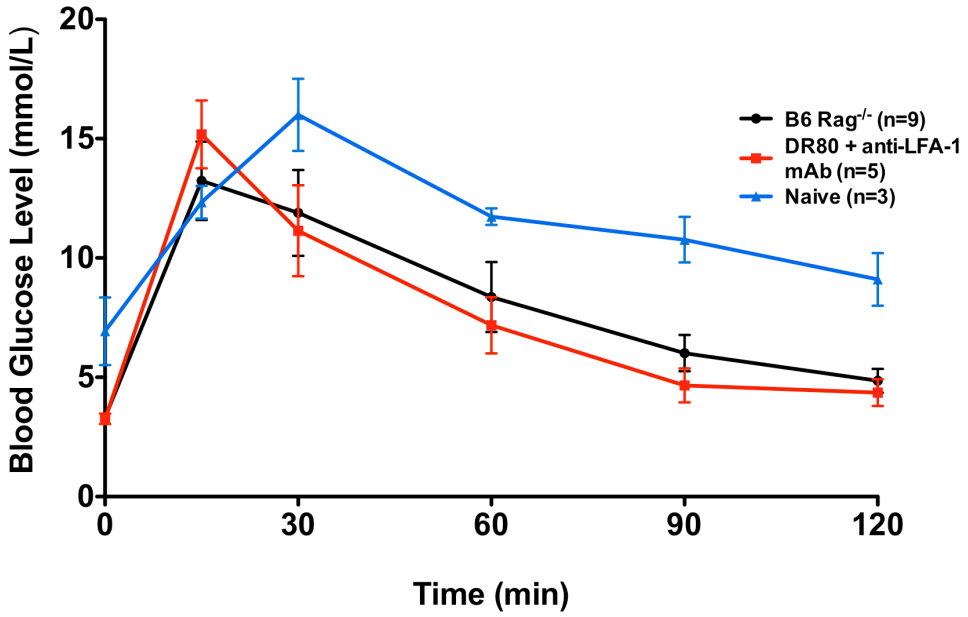

Figure 2-9: Oral glucose tolerance tests of normoglycemic mice that had been transplanted with NPI and treated with DR80 and anti-LFA-1 mAb. Responses to glucose challenge of B6 rag$^{-/-}$ mice transplanted with the same islets and naïve mice are compared. There are no significant differences between the groups.

Mice that were treated with DR80 and anti-LFA-1 mAb and achieved normoglycemia underwent an oral glucose tolerance test to demonstrate their function. When compared with naïve B6 mice and B6 rag mice that had been transplanted with the same NPI, there were no significant differences between their blood glucose levels at any time point (Fig. 2-9). There is a trend towards slightly higher blood glucose levels in the naïve B6 mice, which is not unexpected, and actually comparable to previous findings (17).

Histologic analysis of the grafts recovered from mice that achieved normoglycemia demonstrated insulin positive cells in abundance, with less immune cell infiltrate when compared to graft recovered from mice that

were untreated or mice treated with DR80 and anti-LFA-1 mAb that rejected their grafts (Fig. 2-10). Grafts from untreated mice and mice treated with suramin, DR80, or anti-LFA-1 mAb alone all demonstrated significant cellular infiltrate with no insulin positive staining cells.

When grafts are stained for immune cell subsets, it is obvious that all grafts had significant $CD4^+$ T cell infiltrate (Fig. 2-11). $CD8^+$ T cell infiltrate was much less than $CD4^+$ T cells in all grafts, and was slightly less in grafts that were protected by the treatment with DR80 and anti-LFA-1 mAb. $CD19^+$ cells (B cells) were present in large numbers in all grafts, and were reduced in protected grafts (Fig. 2-11, 2-12). These findings, when taken together, suggest that $CD4^+$ T cells are very important in the rejection of NPI xenografts, more so than $CD8^+$ T cells. B cells also appear to be very important in the rejection of the grafts, and treatment with DR80 and anti-LFA-1 mAb decreases the amount of cellular infiltrate.

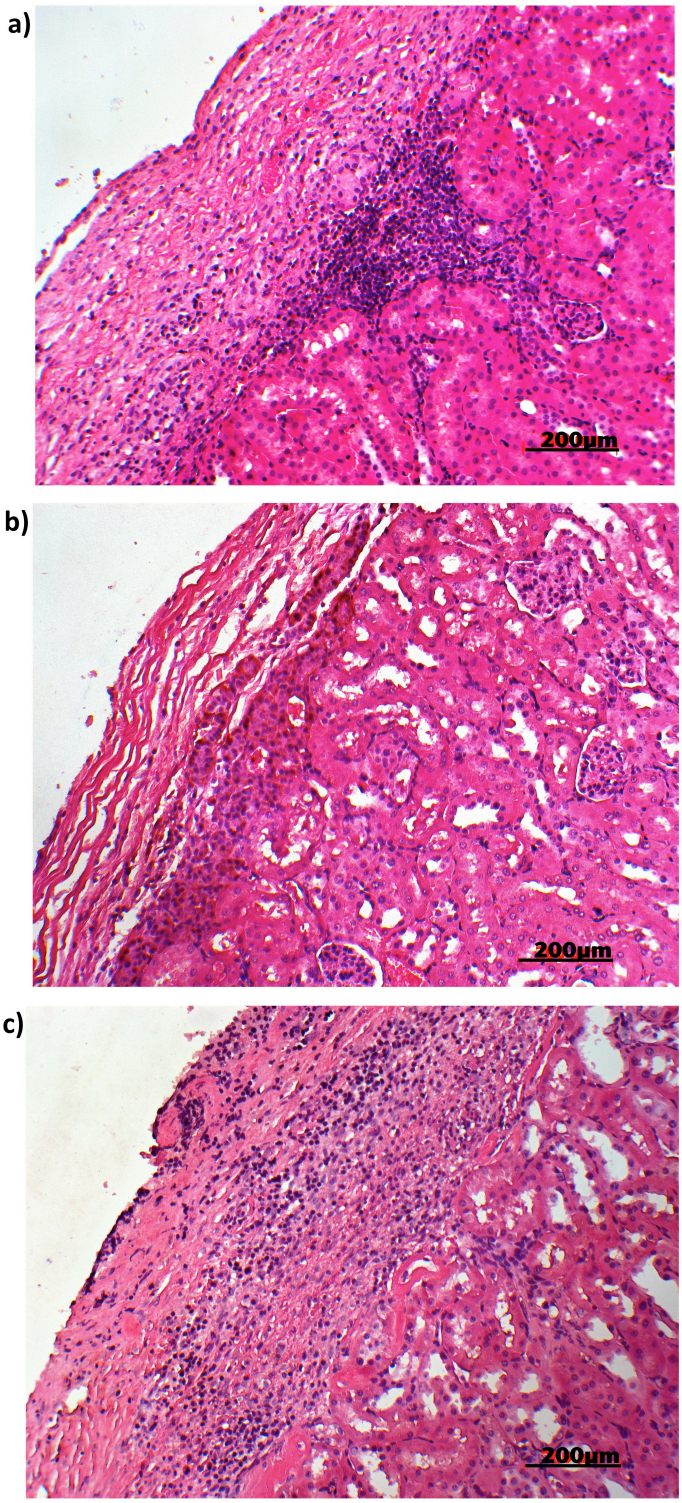

Figure 2-10: Immunohistochemical analysis of grafts recovered from a) an untreated mouse, b) a mouse treated with DR80 and anti-LFA-1 mAb that achieved normoglycemia, and c) a mouse treated with DR80 and anti-LFA-1 mAb that rejected its graft. Brown staining indicates insulin positive cells.

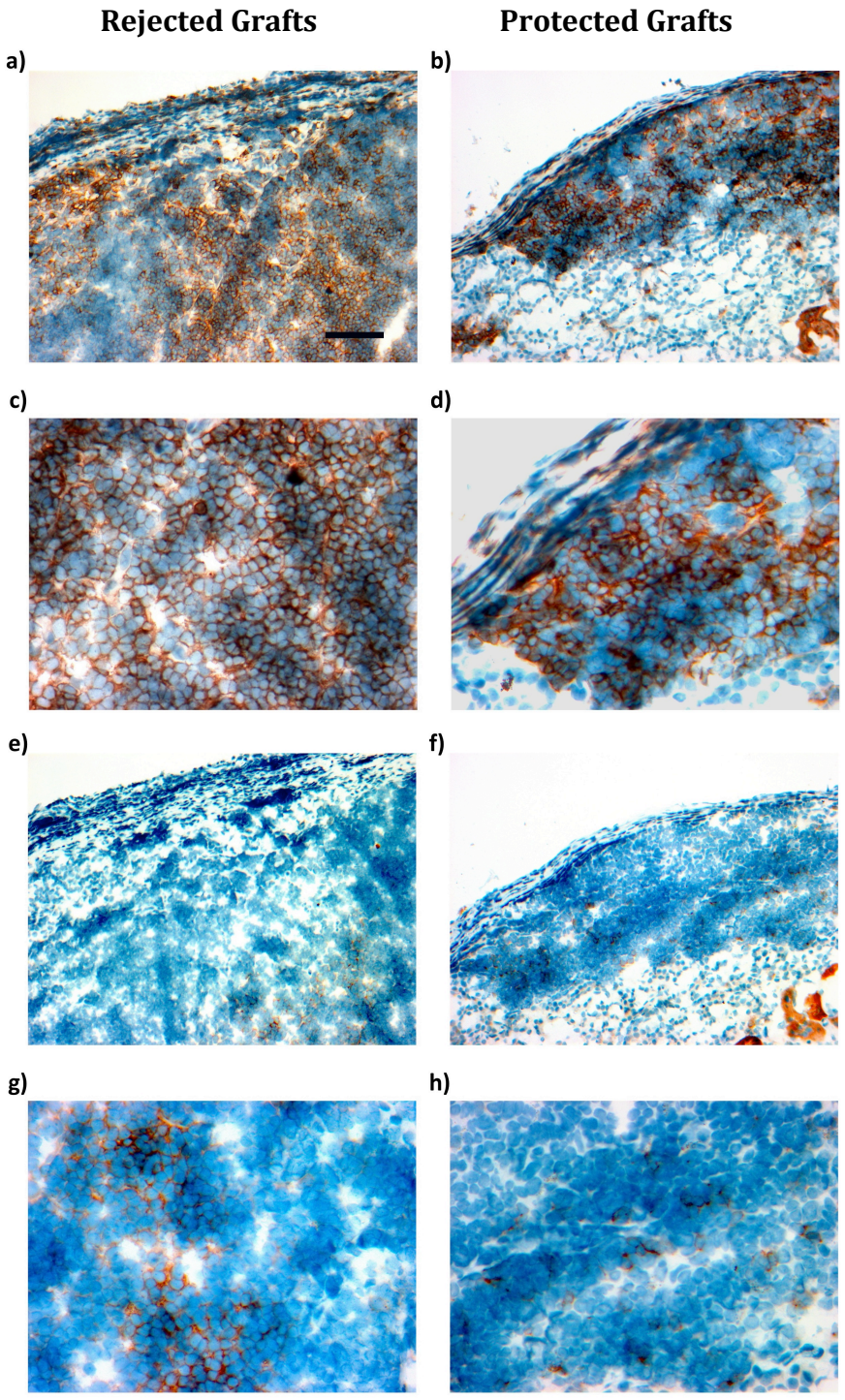

Figure 2-11: Immunohistochemical analysis of grafts recovered from mice treated with DR80 and anti-LFA-1. Sections a) through d) show staining for $CD4^+$ cells, while e) through f) show staining for $CD8^+$ cells. Sections a), b), e), and f) are 10x magnification; sections c), d), g), and h) are 25x magnification. Bar indicates 200µm and 500µm in 10x and 25x sections, respectively.

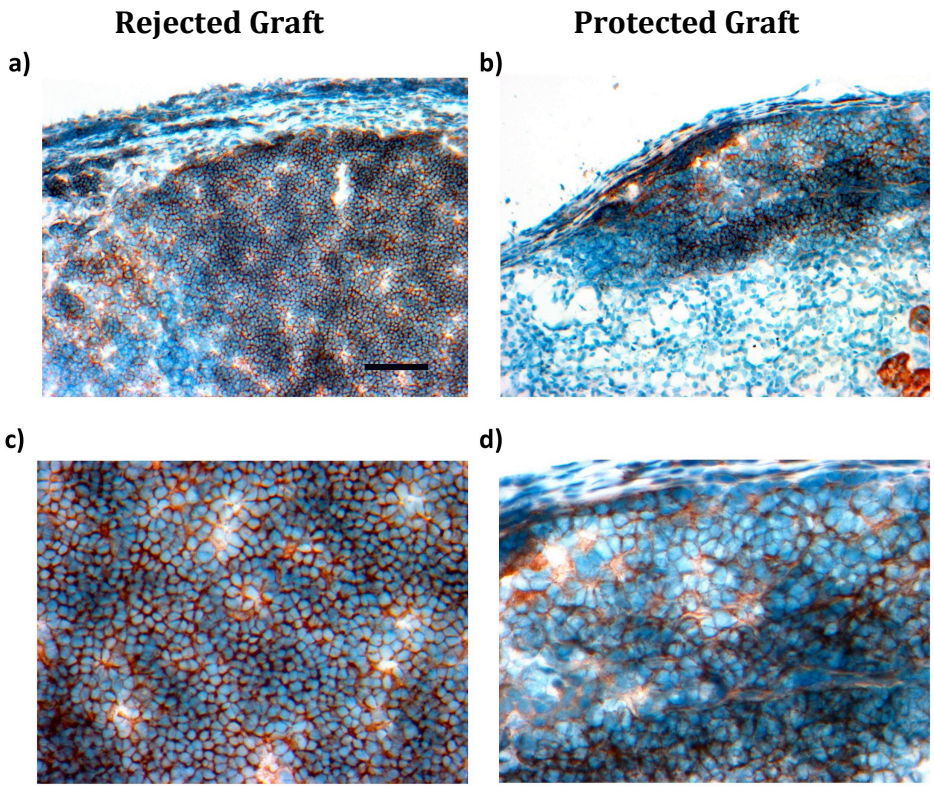

Figure 2-12: Immunohistochemical analysis of grafts recovered from mice treated with DR80 and anti-LFA-1 mAb. Brown staining represents $CD19^+$ cells. Sections a) and b) are 10x magnification; sections c) and d) are 25x magnification. Bar indicates 200µm and 500µm in 10x and 25x sections, respectively.

DR80 + anti-LFA-1 mAb: Rejected	DR80 + anti-LFA-1 mAb: Protected	Naïve
8.18	12.22	3.88
61.69	3.54	3.95
	5.45	4.66

Table 2-3: Percent of porcine splenocytes bound to anti-porcine IgG in mouse serum. Porcine splenocytes were incubated with diluted mouse serum (1:128) for 1 hour then washed with PBS and incubated with goat anti-mouse IgG FITC labeled antibody for 1 hour. Flow cytometry was used to detect the percentage of live splenocytes bound to IgG. Although there is an indication that mice that achieved normoglycemia had a reduced humoral response to the porcine tissue, the number of available samples for this assay limits the conclusions that can be drawn from this data.

The determination of anti-porcine Ab in the serum of the treated mice was limited by the availability of the porcine splenocytes in order to perform the assay. Therefore only limited data could be collected. There does appear to be a lower level of anti-porcine IgG Ab in the serum of mice that achieved normoglycemia, approaching that of naïve mice (Table 2-3). There is one outlier, however, in that a mouse that rejected its graft still had a low level of Ab in its serum. This suggests it is not only the humoral response that is important in the rejection of the NPI xenograft, however, as stated above, limited conclusions can be drawn from this data.

2.3.4 Protection induced by DR80 and anti-LFA-1 mAb may be due to an increase in T regulatory Cells

The spleen cells from all mice were isolated and analyzed by flow cytometry to characterize the immune cell phenotype. Although no differences were significant between mice with a protected islet xenograft and mice that rejected the islet xenograft or naïve mice, there were some trends towards an increase in markers of T regulatory cells in the mice with protected grafts (Fig. 2-13). These markers include Cytotoxic T-lymphocyte antigen 4 (CTLA4), a cell surface molecule found on helper T cells and regulatory T cells. It binds to B7 molecules (CD80, CD86) and transmits inhibitory signals (28). It also includes Foxp3 which is considered to be master regulator in the development and function of regulatory T cells (28). The trend towards an increase in these markers on $CD4^{+}CD25^{+}$ T cells in mice that achieved normoglycemia after being treated with DR80 and anti-LFA-1

mAb suggests a possible role of T regulatory cells in the protection of the islet xenografts.

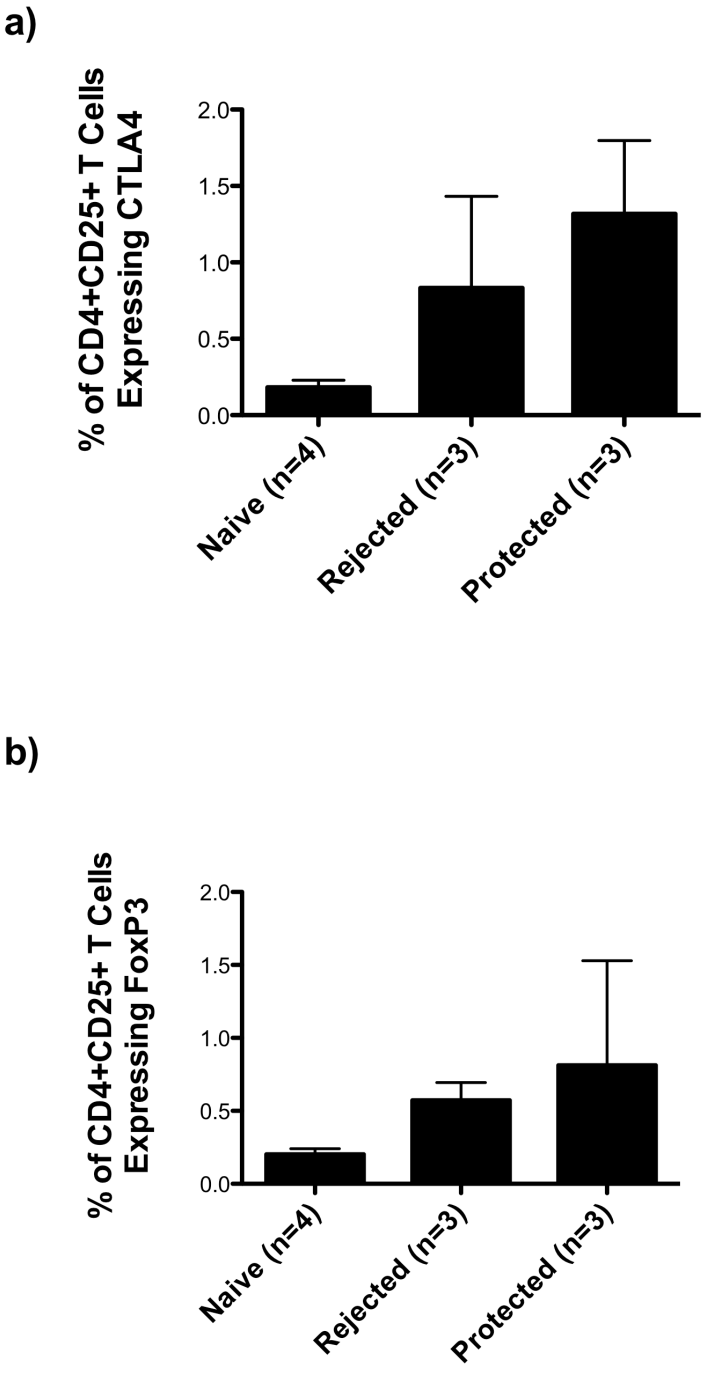

Figure 2-13: Percent of $CD4^+CD25^+$ T cells expressing CTLA4 and Foxp3 in naïve B6 mice or B6 mouse recipients of NPI treated with DR80 and anti-LFA-1 mAb that rejected or accepted the NPI xenografts.

2.4 DISCUSSION

It has been previously reported that the small inhibitory molecules suramin and DR80 are capable of inhibiting the CD40-CD154 co-stimulatory interaction *in vitro*, in both cell free binding assays and B cell proliferation assays (25,26). The aim of the present study was to confirm these findings and to determine the *in vivo* effect of these compounds on the rejection of NPI xenografts in mice.

Initially, we performed *in vitro* toxicity assays of suramin and DR80, as well as several conventional immunosuppressive medications, on NPI. Minimal cytotoxicity was demonstrated with either suramin or DR80, and in fact, they were less cytotoxic to NPI than conventional immunosuppressive medications currently being used clinically. As the toxic effects of immunosuppressive drugs on β cells may contribute to graft failure, these compounds show promise as alternative treatments that do not have this same clinical concern.

Next we examined the ability of suramin and DR80 to inhibit T cell proliferation *in vitro.* Non-antigen specific T cell proliferation was inhibited in a dose dependent manner, with IC50's similar to that of previous studies involving non-antigen specific B cell proliferation (25,26). Antigen-specific T cell proliferation required higher concentrations of the compounds in order to be inhibited. Inhibition in both settings cannot be accounted for by cytotoxicity, as neither suramin nor DR80 exhibited significant toxicity to

murine splenocytes at any of the concentrations studied. As previous studies have demonstrated the *in vitro* specificity of these compounds to the CD154 molecule (25,26), we can therefore conclude that T cell proliferation was inhibited due to this interaction. This has important clinical implications, as their specificity may allow for the specific blockade of the CD40-CD154 interaction, which may contribute to tolerance to NPI xenografts, without the thromboembolic complications seen with the anti-CD154 mAb.

With regards to *in vivo* studies, suramin had very little success in the prevention of NPI rejection in mice. As monotherapy, none of 6 mice treated achieved normoglycemia, and 5 had to be euthanized due to poor health because they were diabetic. When combined with anti-LFA-1 mAb, only 1 of 5 mice achieved normoglycemia, while 3 of the 5 had to be euthanized due to poor health induced by diabetes. It was concluded that suramin displayed significant toxicity, therefore no additional animals were tested.

For the first time, DR80 was administered *in vivo* in an attempt to prevent NPI xenograft rejection. Although monotherapy with DR80 was not effective, the combination of DR80 with anti-LFA-1 mAb led to an increase in the survival of NPI xenografts. Six of 10 mice treated with this combination therapy achieved normoglycemia within 16-25 weeks. This is an improvement upon the previously documented rate of success with anti-LFA-1 mAb alone, which had a success rate of <50% (17). One mouse did reject shortly after achieving normoglycemia, approximately 185 days post-transplantation. This treatment did not however, achieve the 100% success

rate of long-term NPI survival that Arefanian *et al.* had found when anti-CD154 and anti-LFA-1 mAbs were combined (17). Therefore although the combination of DR80 and anti-LFA-1 mAb improved upon monotherapy with anti-LFA-1 mAb, it was inferior to the combination of anti-LFA-1 with anti-CD154 mAb.

The immune cell phenotypes of mice treated with DR80 and anti-LFA-1 mAb that were protected, as well as those that rejected, were compared with that of naïve mice. No statistically significant results were found, however, there were trends towards an increase in markers of T regulatory cells in the mice that achieved and maintained normoglycemia. Both CTLA4 and Foxp3 were increased in $CD4^+CD25^+$ T cells. CTLA4 transmits an inhibitory signal to T cells, while Foxp3 is the master regulator of T regulatory cells (28). Arefanian *et al.* had found an increase in these markers in tolerant mice that had been treated with anti-LFA-1 mAb and anti-CD154 mAb and transplanted with NPI (17). These results are from a larger number of transplanted mice (17), therefore it is possible that with an increase in the number of mice in these studies, we may too yield significant results.

Immunohistochemistry displayed a decreased amount of immune cells infiltrating the graft in mice that achieved normoglycemia, while demonstrating robust insulin staining. All grafts displayed significant $CD4^+$ and $CD19^+$ infiltrate, although this was decreased in protected grafts of mice treated with DR80 and anti-LFA-1 mAb. $CD8^+$ T cells were in much less abundance compared with the other cell types. These results ultimately

suggest that $CD4^+$ T cells are very important in the rejection of NPI, more so than $CD8^+$ T cells. This has been demonstrated in the past, and may be due to the indirect antigen presentation utilized in discordant xenograft models (29,30,31). B cell infiltration into the graft, as well as the anti-porcine IgG Ab response, both seem to be modulated by the treatment of DR80 and anti-LFA-1 mAb. This is in keeping with other studies, which have demonstrated that costimulatory blockade is able to decrease immune cell infiltrate and modulate antibody response (17,27), and with *in vitro* studies of DR80 which has shown that B cell proliferation and activation is decreased (25,26).

Our studies demonstrate for the first time the ability of suramin and DR80 to inhibit T cell activation *in vitro* with minimal cytotoxicity. They also demonstrate the potential of DR80 to prevent NPI xenograft rejection in mice, in combination with anti-LFA-1 mAb. DR80 is an interesting compound that will need to be investigated further in order to determine its therapeutic potential.

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CHAPTER 3

GENERAL DISCUSSION AND CONCLUSIONS

3.1 GENERAL DISCUSSION

Type 1 DM is a chronic disease due to the absence of insulin production by the pancreatic β cells (1,2). Vigilant blood glucose monitoring and exogenous insulin therapy are required to maintain tight physiologic glycemic control. This tight regulation is extremely important as it may prevent the onset and progression of the secondary complications of type 1 DM, including retinopathy, nephropathy, and even heart attacks and strokes (3,4). There are multiple factors that alter the glycemic levels in the body at any given moment, and the reactive nature of insulin therapy is often not sufficient to maintain physiologic glucose levels. Intensive insulin therapy can provide stricter control, however the incidence of hypoglycemic episodes is greatly increased in these patients (4). These episodes are particularly dangerous as they may lead to coma, seizures, or death. The so-called "brittle" diabetics have even greater glycemic lability and are therefore even more prone to these hypoglycemic episodes (3,4). Therefore, there needs to be a more physiologic approach to the treatment of type 1 DM in order to prevent the complications of the disease and to ensure a long and healthy life in affected individuals.

Although a number of strategies are being examined in order to achieve physiologic maintenance of glucose levels, the most realistic of the

options is islet transplantation. The success of the Edmonton Protocol in 2000 brought this modality into the spotlight, as 7/7 patients who received islet allografts achieved and maintained normoglycemia for at least one year post-transplantation (5). Glucose control was greatly improved and hypoglycemic episodes were reduced (5). The toxicity of the immunosuppressive regimen, as well as the shortage of cadaveric donors for transplantation, currently limits this procedure to a small subset of individuals with type 1 DM (6,7). In order to facilitate the widespread use of islet transplantation, a safe and effective anti-rejection regimen, as well as an alternative source of islets, will need to be developed.

The shortage of donor tissue can be solved in the near future with the use of xenogeneic tissue. Porcine tissue is the most promising islet source, as the pig donors breed rapidly, have large litters, can be housed in pathogen free environments, and can be genetically altered to create safer donor tissue for transplantation (8-11). Porcine and human islets are morphologically and physiologically similar to each other, and porcine insulin has been used in the treatment of type 1 DM for years (8-11). Both neonatal and adult porcine tissue has been effective in establishing euglycemia in pre-clinical NHP models (12-15), demonstrating that these islets have the potential for clinical use. Neonatal porcine islets, however may provide the most practical solution. They are housed for less time therefore incur less cost. In addition, they are more resistant to hypoxic damage, have growth potential, and may be less immunogenic (9,15-17). Xenogeneic tissue, however, induces a very

strong cellular immune response by the host, causing loss of tissue and destruction of the graft (9,18). In order to prevent rejection of the xenograft, without the use of harmful immunosuppressive medications, a short-term therapy with the ability to induce tolerance to the donor tissue with minimal toxicity is required. It is when this is developed that islet xenotransplantation may be clinically applicable.

The specific targeting of important biologic pathways in T cell activation and function may provide this solution. Two of these pathways are LFA-1 binding with ICAM-1 and CD40 binding with CD154. Leukocyte function antigen-1 (LFA-1) has a number roles involved in the adhesion, activation, and migration of leukocytes (19-24). Binding of LFA-1 to ICAM-1 has been shown to lower the antigen requirements necessary for T cell activation, optimize contact between the T cell and its APC, and provide co-stimulatory signal to T cells (19-24). Due to its many functions, blockade of this interaction can lead to tolerance. In concordant rat to mouse islet xenotransplantation, monotherapy with an anti-LFA-1 mAb was highly effective in preventing rejection (25). However, in discordant pig to mouse islet transplantation, it was only moderately effective when used as monotherapy (26).

The CD40 interaction with CD154 is another potential target as it too has a variety of functions that are important in the activation of both cytotoxic T cells and helper T cells. It has been shown to up-regulate co-stimulatory molecules on APC, up-regulate IL-2 receptors on T lymphocytes,

and promote the release of various cytokines (27-32). The blockade of this interaction with an anti-CD154 mAb has proven to be efficacious in promoting long-term allograft survival in rodent and NHP models (33,34).

The combination of the two aforementioned mAb provides even more exciting results. The short-term administration of this regimen allowed for 50/50 diabetic mice transplanted with NPI to achieve normoglycemia, the majority maintaining this long term (26). The anti-CD154 mAb, however, has been shown to increase the incidence of thrombo-embolic events in humans and NHP, thereby halting clinical trials (33-35). The administration of heparin with this therapy has reduced the number of events, however, the rate is still above acceptable limits (35). Activated platelets express CD154 as well, and it is possible that the binding of the Ab may prolong the pro-aggregatory effect of the platelet via a mechanism involving the Fc domain (36).

An alternative to the anti-CD154 mAb that may provide the specific blockade of the CD40-CD154 interaction, without the thrombophilic state that is created by the mAb, is being sought (37-40). This may be in the form of small inhibitory molecules, specifically suramin and DR80, which have been shown to inhibit the CD40-CD154 interaction *in vitro* (38-40). In both cell free binding assays and B cell proliferation assays, these compounds inhibited binding in a dose dependent manner with minimal cytotoxicity (38-40).

In our study, we sought to determine if suramin and DR80 could inhibit the proliferation of murine T cells *in vitro* and if this inhibition could translate into the *in vivo* protection of NPI xenografts in mice. These compounds were examined in combination with the anti-LFA-1 mAb, which has been shown to interfere with T cell activation as well as inhibit humoral responses (23,25,26). In addition, they have been known to facilitate protection of islet xenografts by regulatory T cell subsets (26).

The inhibition of T cell proliferation was accomplished by both suramin and DR80 *in vitro*. These compounds inhibited T cell proliferation in a dose dependent manner with minimal cytotoxicity. Non-antigen specific proliferation was inhibited in concentrations similar to what was previously found for non-antigen specific B cell proliferation (38-40). Antigen specific proliferation, however, required higher concentrations in order to achieve the same inhibition. This is the first known examination of the inhibition of antigen-specific T cell proliferation with these compounds.

As monotherapy, neither suramin, DR80, nor anti-LFA-1 mAb were able to protect the NPI xenografts *in vivo*. Suramin failed to provide significant graft protection even in combination with anti-LFA-1 mAb. The synergistic effect of DR80 and anti-LFA-1 mAb appears to be more efficacious in preventing NPI rejection than either of these 2 therapies alone. Six of 10 mice with this treatment achieved normoglycemia, although one subsequently rejected. The antibody response of the mice that achieved normoglycemia appears to be significantly reduced compared with mice that

rejected, suggesting that this therapy targets the humoral response. $CD4^+$ T cells made up a significant portion of the cellular infiltrate, suggesting that they are important in the rejection of NPI xenografts in this discordant model. This confirms previous findings and is likely due to the reliance on indirect antigen presentation (45,46). $CD19^+$ B cells were also present in significant numbers and did decrease in protected grafts. However, the presence of significant immune cell infiltrate even in normoglycemic mice, and the 60% rate of conversion to normoglycemia, suggests this protection is incomplete.

Flow cytometric data demonstrated a trend towards an increase in the expression of CTLA4 and Foxp3 by $CD4^+CD25^+$ T cells in mice treated with DR80 and anti-LFA-1 mAb that achieved normoglycemia. CTLA4 is responsible for sending inhibitory signals to T cells, while Foxp3 is the master regulator of T regulatory cells (47) and cells positive for this marker has been previously shown to be present in protected grafts (26). The increased expression of these molecules suggests a role for T regulatory cells in the protection of these grafts. These differences, however, were not statistically significant, so conclusions regarding the mechanism of protection cannot be drawn at this time. It will be prudent to repeat these studies with a larger number of transplanted animals in order to determine if this proposed mechanism is indeed true.

Despite the aforementioned results, there still remain a number of important issues that need to be addressed, including: *i)* the purification of

DR80; *ii)* the pharmacokinetics of DR80; *iii)* the mechanism of protection; and *iv)* the safety and efficacy of this compound in more clinically relevant models, for example large animal and autoimmune transplant models.

DR80, as it is commercially available at this time, is relatively impure, with a dye content of ~25% (41). It is conceivable that its efficacy in a more pure form would be increased significantly, while at the same time decreasing its toxicity *in vivo*. High performance liquid chromatography (HPLC) is a possible mechanism to purify the compound for clinical use. Whether this will be effective in increasing the efficacy of the compound in preventing NPI xenograft rejection will need to be determined by repeating the experiments with the purified product.

As this is the first attempt at the use of DR80 *in vivo*, there is very little known in terms of its pharmacokinetics. The half life and route of elimination will need to be determined, and this is in the plans for future studies. Serial sampling of murine serum after injection with DR80 will allow for determination of the half-life of the compound, while urine and liver analyses may provide some insight into the route of elimination. Until these studies are performed, proper dosing cannot be established, and safety profiles are virtually unknown. It is possible that by optimizing the dose of the dye, we may be able to optimize the regimen and achieve improved results.

Although there is suspicion that T regulatory cells are involved in the protection of NPI xenografts after treatment with DR80 and anti-LFA-1 mAb, the exact mechanism of protection is yet to be determined. Further studies

will need to be performed including the determination of cytokines involved as well as further characterization of the cellular and humoral response seen in animals treated with this therapy. By characterizing the response, we may be able to better identify the cells and molecules involved in tolerance, which may then lead to more appropriate therapies.

It would also be important to determine if the combination of DR80 and anti-LFA-1 mAb leads to the development of tolerance rather than short-term graft protection. In tolerance, the immune system is not reactive to the NPI graft whereas in graft prolongation, the rejection process is merely postponed. If tolerance is induced, graft protection should be indefinite; therefore longer-term studies will need to be performed in order to prove this. In addition, it needs to be determined whether the specificity of the tolerance is donor vs. islet vs. pig specific unresponsiveness. Currently, many recipients of islet transplantation require a second, or even third, islet transplantation in order to maintain normoglycemia. It is prudent to know if each subsequent transplant requires a new course of therapy, or if the tolerance developed is extended to the new graft as well.

Finally, the safety and efficacy of these treatments need to be determined in a more clinically translatable transplant model. The safety profile of anti-LFA-1 mAb is somewhat established as it has been used in the treatment of psoriasis (42). Although there is concern regarding the development of progressive multifocal leukoencephalopathy (PML), this disease remains quite rare, and is associated with long-term use of the

medication (42-44). However, its safety in islet transplantation needs to be determined. Further, the safety of DR80 will need to be examined. As a class, azo dyes have been identified as carcinogenic, however little is known about DR80 in particular (41). It is possible the purified form of DR80 may be less harmful than the currently commercially available impure form. The administration of these compounds in large animal models may reveal any concerns regarding their use and ultimately determine their clinical applicability. Further to this, an autoimmune diabetic animal model will determine the efficacy of these treatments in a setting more relevant to individuals with type 1 DM.

Overall, the results of our study indicate that the small inhibitory molecules suramin and DR80 are capable of inhibiting T cell proliferation *in vitro* in a dose dependent manner, with minimal cytotoxicity. In addition, the combination of DR80 and anti-LFA-1 mAb is moderately effective in prolonging NPI xenograft survival in a diabetic mouse model, and is superior to either of these compounds alone. Further studies regarding the pharmacokinetics, safety profile, and mechanism of protection of these molecules will need to be performed.

3.2 CONCLUSION

Islet transplantation is the most promising treatment for type 1 DM due to the potential to restore physiologic control of glucose levels. This control translates much more tangible results for the patient - eliminating the need to constantly check blood glucose levels and inject themselves with

insulin, and the prevention of devastating secondary complications that can leave them blind, on dialysis, or even dead.

There are two major barriers that have prevented islet transplantation from becoming a widespread clinical therapy: *i)* the shortage of donor tissue for transplantation; and *ii)* the requirement for the chronic use of harmful immunosuppressive drugs. The first barrier may be solved by the use of xenogeneic tissue, in particular NPI, however its clinical use is dependent upon the development of safe and effective anti-rejection therapies.

In these studies, we have demonstrated that the small inhibitory molecules suramin and DR80 are capable of inhibiting T cell proliferation *in vitro*. DR80, when used in combination with anti-LFA-1 mAb, is moderately effective in preventing the rejection of NPI xenografts. As this is the first *in vivo* effort using DR80, more studies will need to be performed in order to confirm this data and to further characterize the pharmacokinetics of the compound as well as the mechanism of protection. It is our hope that this data may provide the basis for further research and ultimately contribute to the development of safe anti-rejection therapies for islet xenografts.

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