University of Alberta

Co-transplantation of Neonatal Porcine Islets with Sertoli Cells Combined with Short-term Monoclonal Antibody Therapy in Preventing Neonatal Porcine Islet Xenograft Rejection

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Experimental Surgery

Department of Surgery

Fall 2009

Edmonton, Alberta

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I would like to dedicate this thesis to the three people who inspire me most, my parents Rozina and Alnasir Ramji and my sister Nabeela, whose love, guidance, and support have made me better in every way

ABSTRACT

The need for an unlimited source of islets and a safer method of immunosuppression has limited the widespread application of islet transplantation. To remedy the shortage of donor tissue, xenotransplantation of neonatal porcine islets (NPI) has been proposed. In this study we sought to determine if combining co-transplantation of NPI with Sertoli cells (SC) with a short-term monoclonal antibody (mAb) therapy would prevent NPI xenograft rejection. We hypothesize that this combination of treatments will lead to long-term NPI xenograft survival.

Our result show a significant increase in the proportion of mice achieving long-term graft survival compared to untreated mice transplanted with NPI alone, as 7/7 mice treated with anti-LFA-1 mAb (p=0.001), 7/8 mice treated with anti-CD154 mAb (p=0.003), and 4/9 mice treated with anti-CD45RB mAb (p=0.020) achieved and maintained normoglycemia long-term. Therefore, we conclude that the combination of mAb therapy with SC is highly efficacious in preventing NPI xenograft rejection.

ACKNOWLEDGEMENTS

My graduate training has been an exciting period of my life and could not have been accomplished if not for the support and generosity of others. I would first like to acknowledge the members of my examining committee, Dr. Gina Rayat, Dr. Ray Rajotte, Dr. Babita Agrawal, and Dr. Catherine Field for their careful review and reflection of the work I have done during my Master's training. My Master's work has been important to me and I genuinely appreciate their participation and effort in helping me to become a better student.

To my Supervisor Dr. Gina Rayat, I want to thank you for being an amazing mentor and for all the support and encouragement you have provided over the past two years. You have pushed me to be a more able and confident researcher and person, and I am genuinely proud to have had the opportunity to have been your student. I would also like to thank Dr. Ray Rajotte, Dr. Ron Gill, Dr. Thomas Churchill, and Dr. Greg Korbutt for taking a special interest in my work and in my career goals. You have all been great mentors and I truly appreciate all your generosity and kindness.

To my mentors in the lab, Hossein Arefanian, Dr. Dereck Mok, and Eric Tredget, thank you so much for making my Master's training so amazing. Hossein has been an incredible help in the lab and truly epitomizes what it means to be a dedicated researcher, father, husband, and friend. I am definitely going to miss working with him and his unparalleled coffee. Both Dereck and Eric have also

been extraordinary mentors and have been two people I could look up to and have always been there to provide advice and friendship. I would also like to thank Deb Dixon (also known as Mouse Lady) for her help and for just being an awesome person. I have had so much fun learning from Deb and working with her and I can say with the utmost confidence that my graduate training would not have been as fun and productive if not for her. Thank you also to James Lyon for being a technician with some attitude and for his assistance throughout my graduate training.

I would also like to extend my appreciation to Colleen Ruptash, Michelle Maccagno, and Rosemarie Henley who not only helped me weave through all the administrative tasks required for me complete my Master's degree, but also brightened my days while doing it.

Lastly, thank you to my friends and family. There are certain people that you will never be able to repay in life and my parents are at the top of this list for me. I love you both so much, and I want to thank you for your love, support, generosity, and guidance. I am truly blessed to have you as my parents.

I am honored to have had the opportunity to work with all of you, and I wish you all the very best in all your future endeavors.

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LIST OF ABBREVIATIONS

ABC	Avidin-biotin complex
ALS	Anti-lymphocyte serum
APC	Antigen presenting cells
ATP	Adenosine triphosphate
B6	C57BL/6
BSA	Bovine serum albumin
BTLA	B and T lymphocyte attenuator
CD	Cluster of differentiation
ConA	Concanavalin A
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
DAB	3,3-diaminobenzidinetetrahydrochloride
DM	Diabetes mellitus
EGF	Epidermal Growth Factor
EGTA	Ethylene glycol-bis (2-aminoethyl)-N,N,N',N'-tetraacetic acid
FACS	Florescence activated cell sorter
FasL	Fas ligand
Foxp3	Forkhead box P3
GMP	Good manufacturing practice
HBSS	Hank's balanced salt solution
HLA	Human leukocyte antigen
IBMIR	Instant blood mediated inflammatory reaction
ICAM	Intercellular adhesion molecule-1
IFG-γ	Interferon-gamma
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
JAK	Janus kinase
JAM-1	Junctional adhesion molecule-1
LFA-1	Leukocyte function associated antigen-1
mAb	Monoclonal antibody
MAC	Membrane attack complex
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
MIS	Müllerian inhibiting substance

NF-κB	Nuclear factor κB
NK	Natural killer
NOD	Non-obese diabetic
NPI	Neonatal porcine islets
OCT	Optimal cutting temperature
PBS	Phosphate buffered saline
PD-1	Programmed death-1
PD-L1	Programmed death-1 ligand
PERV	Porcine endogenous retrovirus
SC	Sertoli cells
SCID	Severe combined immunodeficiency
Serpina3n	Serine proteinase inhibitor-3
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
T_H	T helper
TNF-α	Tumor necrosis factor-alpha
TRAF	Tumor necrosis factor receptor-associated factor

CHAPTER 1

GENERAL INTRODUCTION

1.1 DIABETES MELLITUS

Diabetes mellitus (DM) constitutes a heterogeneous group of metabolic disorders that share a common feature of hyperglycemia [1]. This hyperglycemic condition can be attributed to a reduction in the secretion and/or action of insulin [1,2]. As a result, patients of this chronic condition suffer from abnormalities in fat, sugar, and protein metabolism resulting in a myriad of complications including dysfunctions in organs such as the kidneys, eyes, nerves, heart, and blood vessels [2]. Clinical symptoms include polyuria, polydipsia, fatigue, polyphagia, weight loss, blurred vision, and persistent hyperglycemia [3]. Patients may also suffer from growth impairments and may have an increased susceptibility toward developing secondary infections [4].

Acute life-threatening complications of uncontrolled diabetes include hyperglycemia with ketoacidosis or non-ketotic hyperosmolar syndrome, whereas long-term complications include: *i)* retinopathy with potential vision loss; *ii)* nephropathy leading to kidney failure; *iii)* peripheral neuropathy leading to amputation, foot ulcers, and Charcot joints; and *iv)* autonomic neuropathy leading to genitourinary, gastrointestinal, and cardiovascular symptoms, as well as sexual dysfunction [4].

1.1.1 Impact and Cost of Diabetes

It is currently estimated that approximately 246 million people suffer from diabetes worldwide, a number that expected to rise to 380 million by 2025 [5]. This rate of increase is also seen in Canada where the number of patient with diabetes is expected to increase from the 2 million current patients to approximately 3 million patients by 2010 [5]. In the United States, diabetes mellitus is the leading cause of end-stage renal disease, non-traumatic lower extremity amputations, and adult blindness [1]. It is also the fifth leading cause of death worldwide, claiming nearly 3 million lives annually [1]. Diabetes has also had a major impact on the health care system as diabetes related expenses are expected to cost the Canadian healthcare system $15.6 billion dollars in 2010 and is expected to reach $19.2 billion by 2020 [5]. Even more, diabetes related healthcare expenses in the United States in 2005 were estimated to be $100 billion [6].

At a personal level, diabetes can result in significant morbidity and places heavy demands on patients as it necessitates continuous monitoring of blood glucose levels and potentially daily injections of exogenous insulin. The management of this condition becomes even more onerous in the face of constant fluctuations in blood glucose levels associated with food intake, activity levels, stress levels, and other factors that invoke changes, adding further limitations to patients in their daily lives. Direct patient costs for treating diabetes are estimated to range from $1,000 to $15,000 per year in Canada [5]. These factors, combined with the debilitating secondary complications associated with the disease can greatly impair the quality of life of patients suffering from diabetes.

1.1.2 Classification of Diabetes

Though many subgroups exist, majority of patients with DM fall within 2 major etiopathogenetic categories; type 1 and type 2 DM. Type 1 DM, previously known as juvenile diabetes or insulin dependent DM, can be further classified within two groups based on serological evidence of autoimmunity. Type 1a is believed to result due to an immune-mediated destruction of the β cells within the islets of Langerhans, resulting in a complete absence of insulin secretion [3]. In contrast, patients with type 1b have no evidence of autoimmunity but still demonstrate an absence of β cells and thus this group of conditions is classified as idiopathic [3]. Type 2 diabetes, previously known as non-insulin dependent DM, is caused by a combination of insulin resistance and an inadequate compensatory insulin secretory response [3]. While this form of diabetes is much more prevalent than type 1 diabetes, its clinical symptoms are generally much less severe, with patients often not needing insulin injections for survival. The risk of type 2 DM is increased with age, obesity, and sedentary lifestyle [7].

Gestational diabetes is another form of diabetes which occurs in pregnant females and is characterized by insulin resistance. This form of diabetes affects approximately 4% of pregnant females [1]. Other etiologies of diabetes included genetic defects in β-cell function, genetic defects in insulin action, diseases of the exocrine pancreas, endocrinopathies, drug or chemical induced diabetes, infections, genetic syndromes associated with diabetes (i.e. Down's syndrome), and other uncommon forms of immune-mediated diabetes [3].

Classifications of Diabetes

I. Type 1 diabetes (β-cell destruction, usually leading to absolute insulin deficiency)
   A. Immune mediated
   B. Idiopathic
II. Type 2 diabetes (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance)
   A. Genetic defects of β-cell function
      1. Chromosome 12, HNF-1α (MODY3)
      2. Chromosome 7, glucokinase (MODY2)
      3. Chromosome 20, HNF-4α (MODY1)
      4. Chromosome 13, insulin promoter factor-1 (IPF-1; MODY4)
      5. Chromosome 17, HNF-1β (MODY5)
      6. Chromosome 2, *NeuroD1* (MODY6)
      7. Mitochondrial DNA
      8. Others
   B. Genetic defects in insulin action
      1. Type A insulin resistance
      2. Leprechaunism
      3. Rabson-Mendenhall syndrome
      4. Lipoatrophic diabetes
      5. Others
   C. Diseases of the exocrine pancreas
      1. Pancreatitis
      2. Trauma/pancreatectomy
      3. Neoplasia
      4. Cystic fibrosis
      5. Hemochromatosis
      6. Fibrocalculous pancreatopathy
      7. Others
   D. Endocrinopathies
      1. Acromegaly
      2. Cushing's syndrome
      3. Glucagonoma
      4. Pheochromocytoma
      5. Hyperthyroidism
      6. Somatostatinoma
      7. Aldosteronoma
      8. Others
   E. Drug- or chemical-induced
      1. Vacor
      2. Pentamidine
      3. Nicotinic acid
      4. Glucocorticoids
      5. Thyroid hormone
      6. Diazoxide
      7. β-adrenergic agonists
      8. Thiazides
      9. Dilantin
      10. α-Interferon
      11. Others
   F. Infections
      1. Congenital rubella
      2. Cytomegalovirus
      3. Others
   G. Uncommon forms of immune-mediated diabetes
      1. "Stiff-man" syndrome
      2. Anti-insulin receptor antibodies
      3. Others
   H. Other genetic syndromes sometimes associated with diabetes
      1. Down's syndrome
      2. Klinefelter's syndrome
      3. Turner's syndrome
      4. Wolfram's syndrome
      5. Friedreichs's ataxia
      6. Huntington's chorea
      7. Laurence-Moon-Biedl syndrome
      8. Myotonic dystrophy
      9. Porphyria
      10. Prader-Willi syndrome
      11. Others
III. Gestational diabetes mellitus (GDM)

Table 1.1: Etiological classifications of DM. Adapted from: Diagnosis and classification of diabetes mellitus. Diabetes Care 27 Suppl 1, S5-S10 (2004).

1.1.3 Type 1 DM

Perhaps one of the most fascinating aspects of type 1 diabetes is the selective destruction of the β cells within the islets of Langerhans while sparing other cell types in the pancreas. This form of diabetes affects approximately 5-

10% of diabetic patients [4]. As mentioned previously, a minority of patients have type 1b DM which does not manifest any evidence of autoimmunity and is termed idiopathic. In contrast, there is direct evidence of autoimmunity in other type 1 diabetic patients demonstrating that immunological, in addition to genetic and environmental factors, play a role in the development of this disease. The end result of this debilitating condition is an inadequate insulin supply and thus, a life-long dependence on exogenous insulin for survival [3]. Some of the most severe cases of type 1 diabetes include patients with high glycemic lability and hypoglycemic unawareness. These patients have large fluctuations in glycemic levels and lack the adequate prodromic symptoms such as sweating, tremors, tachycardia, and anxiety, to predict the onset of hypoglycemic episodes [8]. This can be extremely dangerous and concerning for patients as hypoglycemic episodes can lead to coma, seizures, or fatality [6]. These patients are referred to as "brittle" type 1 diabetic patients and for now have been the primary recipients of islet transplants.

1.1.4 Insulin and Metabolic Dysregulation

Insulin is an essential anabolic hormone that promotes growth, regulates fuel mobilization and storage, and is required for the achievement of metabolic homeostasis. Postprandially, insulin secretion promotes the utilization and storage of fuels within the body via: *i)* suppression of hepatic glucose production (gluconeogenesis and glycogenolysis), lipolysis, and proteolysis; *ii)* increasing the

transport of glucose to adipocytes and myocytes; and *iii)* stimulating glycogen synthesis (glycogenesis) [3,9].

Insulin itself is produced within the β cells of the pancreatic islets of Langerhans [1]. Like many other hormones, insulin is synthesized as a preprohormone and is converted to proinsulin in the rough endoplasmic reticulum of the β cells [9]. In storage, proinsulin is cleaved yielding C-peptide and insulin, which will later be secreted in a 1:1 ratio [9]. Because C-peptide remains in the blood stream for longer periods of time than insulin, it is generally used as a marker for insulin secretion. When glucose levels are high, there is an increase in the production of ATP within the β cells, resulting in an inhibition of the K^+ ATP channels. Consequently, there is an influx of Ca^{2+} into the β cells which results in the activation of the voltage gated Ca^{2+} channels. This increase in intracellular Ca^{2+} stimulates exocytosis of the insulin vesicles, releasing insulin and C-peptide [9].

In type 1 DM, the destruction of the insulin producing β cells results in an absence of insulin production. In this case, the body reacts as if it were in a state of starvation, continuing to increase glucose production through the breakdown of fats and proteins. This is further exacerbated by the unabated production of glucagon and growth hormone which continue to induce endogenous glucose production [2]. Though the blood is replete with glucose, most of this glucose cannot be taken up by cells that require insulin dependent glucose uptake, resulting in urinary excretion of the carbohydrate [2].

As mentioned earlier, insulin also inhibits lipolysis. Therefore in type 1 DM, lipolysis is not suppressed and there is an increase in free fatty acids. This elevated free fatty acid flux increases ketogenesis which eventually results in ketoacidosis due to the acidic properties of the metabolites of this process. This can be fatal as acidosis interferes with many enzymatic processes in the body, can enhance circulatory failure, and can cause cardiovascular collapse and other problems [2]. Protein metabolism is also uninhibited due to the insulinopenia, resulting in muscle wasting/cachexia [2].

1.1.5 Etiology of Type 1 Diabetes

The primary defect in type 1 DM is a deficiency in insulin secretion caused by a cell-mediated autoimmune destruction of the β cells within the islets of Langerhans [3]. Though a great deal about this condition is known, the etiology of type 1 DM awaits full elucidation. It is believed that a combination of genetic, immunologic, and environmental factors contribute to the onset of the disease.

While a genetic component is evident, it cannot fully account for the manifestation of the disease. For example, identical twins only have a 25-50% chance of developing type 1 DM when the other sibling has it, demonstrating that genetics alone are not 100% predictive of the disease state [3]. While there are multiple genes that are associated with type 1 diabetes, the HLA genes on chromosome 6 seem to be the most highly associated with the likelihood of developing the disease [3,10]. Specifically, certain combinations of the HLA class II alleles seem to be either protective or predisposing [4]. For example, there is a

strong correlation between certain DQA and DQB genes and the development of diabetes [4]. DRB genes have also been shown to be predictive of the disease state [4]. Because these HLA genes encode for the MHC molecules required for antigen presentation to $CD4^+$ and $CD8^+$ T cells, it makes sense that the pathogenesis of this disease is the result of immunological processes that are dependent on cell-mediated immunological dysfunctions.

Predisposing and Protective HLA Haplotypes in Type 1 DM

Predisposing			
High Risk			
DR3:	DRB1*0301	DQA1*0501	DQB1*0201
DR4:	DRB1*0401	DQA1*0301	DQB1*0302
DR4:	DRB1*0402	DQA1*0301	DQB1*0302
Moderate risk			
DR8	DRB1*0801	DQA1*0401	DQB1*0402
DR2	DRB1*1501	DQA1*0102	DQB1*0502
Protective			
Strong Protection			
DR2	DRB1*1501	DQA1*0102	DQB1*0602
Weak Protection			
DR4	DRB1*0401	DQA1*0301	DQB1*0301
DR4	DRB1*0403	DQA1*0301	DQB1*0302

Table 1.2: Protective and predisposing HLA haplotypes in type 1 DM.
Adapted from: Prevention of type I diabetes and recurrent β-cell destruction of transplanted islets. Endocr Rev 18, 241-258 (1997).

The primary evidence for autoimmunity in type 1 diabetes includes insulitis and the presence of circulating autoantibodies [10]. Three major autoantigens have been identified and include GAD65, insulin, and certain islet tyrosine phosphatases (ICA512 or IA2 and IA2β) [3,4,10]. It is estimated that >90%

of newly diagnosed type 1 diabetics have one or more antibodies against the specific autoantigens mentioned above. In comparison, only 3.5-4% of non-diabetic patients have these autoantibodies, however this group is at greater risk of developing the disease [3]. Autoantibodies themselves do not seem to be the cause of the condition as adoptive transfer of these autoantibodies does not result in the disease state, whereas transfer of T lymphocytes does [3].

As the genetic contribution towards the development of type 1 DM is not complete, it appears environmental factors play a role as well. These contributors however are not well characterized. Viral infections and dietary factors have both been shown to correlate with the disease. Enteroviruses such as coxsackie B4, rotavirus, rubella, mumps and cytomegalovirus have all inconsistently been associated with type 1 DM [3,11]. Dietary contributors such as prolonged breast feeding can decrease the incidence of type 1 DM whereas early exposure to cow milk has been associated with an increased risk of getting the disease [3,12]. A great interest in the role of vitamin D in the development of both type 1 and type 2 DM has also emerged based on evidence that vitamin D may decrease the risk and/or severity of these conditions [13-15].

1.1.6 Clinical Features and Treatments for Type 1 DM

Type 1 DM generally occurs in patients before the age of 30, however, it can occur at any age [3]. Clinical symptoms include polyuria, polydipsia, fatigue, polyphagia, weight loss, blurred vision, and persistent hyperglycemia [3]. Along with the presentation of typical diabetic symptoms, criteria for diagnosis of

diabetes include a fasting plasma glucose level (defined as no caloric intake for eight hours prior to testing) greater than 7.0 mmol/L (126 mg/dl) with a random glucose level exceeding 11.1 mmol/L (200 mg/dl) [3]. Elevated glycated hemoglobin A1C levels may also be used to confirm pre-existing hyperglycemia [3].

As insulinopenia is central to the diseased state, exogenous insulin is an absolute requirement for patients with type 1 DM. Thus, with the discovery of insulin in 1921 by Banting and Best, type 1 DM has changed from a fatal disease to a chronic condition [2]. Along with patient education, dietary control, and continuous monitoring of glycemic levels, exogenous insulin remains the gold standard treatment for patients with diabetes as it effectively lowers blood glucose levels, reverses acute complications of diabetes, and improves both muscle building and fat storage [16]. Exceptional improvements in the types of insulin and insulin delivery systems have also allowed for stricter control of glycemic levels and an increased specificity of the treatment. Short-acting insulin analogues such as Novorapid, Humalog, and Aprida, as well as long acting preparations such as Levemir and Lantus have allowed for a much more accurate regulation of glycemic levels for both bolus and maintenance requirements [16].

Even with these improvements, it is very difficult to achieve a strict regulation of one's blood glucose levels via exogenous insulin, which is a major limitation. In a landmark study conducted by the Diabetes Control and Complications Trial Research Group, it was shown that intensive therapy or strict regulation of glycemic levels significantly decreased the onset and progression of

secondary complications associated with diabetes such as nephropathy, neuropathy, and retinopathy [17]. The major problem however was that intensive insulin therapy resulted in a two-to-threefold increase in severe hypoglycemia, which is a major concern for patients as it can lead to coma, seizures, or death [17]. This becomes even worse for brittle type 1 diabetic patients who already have an increased tendency toward getting hypoglycemic episodes, as well as patients who despite insulin therapy have inadequate glucose control. Thus, intensive insulin therapy is not suitable for all patients with type 1 DM, predisposing them towards developing future complication. Overall, while insulin therapy remains a life saving therapy for many type 1 patients, a more physiological approach will be necessary in order to prevent future complications of diabetes for all patients suffering from the disease.

1.2 ISLET TRANSPLANTATION

1.2.1 History of Islet Transplantation

Interestingly, clinical islet transplantation preceded the discovery of insulin as, in 1889, Oscar Minkowski discovered that a connection existed between the then fatal diabetes and the pancreas. This was shown by the manifestation of polyuria and glycosuria due to the surgical removal of a dogs pancreas [18]. In 1892, they attempted to reverse the condition of a pancretomized dog through subcutaneous autologous transplantation of pancreatic fragments, reporting a temporary reduction in glycosuria [19]. Only one year later, Watson-Williams and Harsant attempted a similar procedure clinically, treating a fifteen

year old boy with fatal ketoacidosis using pancreatic sheep fragments [20]. With little knowledge of the vigor of xenorejection, this procedure was undertaken without immunosuppression. While there was a temporary improvement in the patient's condition, the graft was ultimately rejected, and the patient died three days later.

The practice of transplanting islets rather than pancreatic fragments was facilitated by improvements in islet isolation techniques. This began with Claus Hellerström who isolated rodent islets using free-hand microdissection [21]. Moskalewski improved the islet yield via mechanical and enzymatic digestion of the pancreas using bacterial collagenase [22]. Lacy and Kostianovsky later improved this digestion technique by cannulating the common bile duct and distending the pancreas with Hank's solution prior to mechanical and enzymatic digestion with collagenase [23]. Lindall et al., later improved islet purity while preserving islet function using differential density elutriation with Ficoll, a polymer of sucrose [24].

With the improvements in the isolation procedure, islet transplantation in rodent models began. Younoszai et al., were the first to successfully transplant islets but only temporary improvements in glycemic levels resulted [25]. Two years later, Ballinger and Lacy reported that 400-600 syngeneic pancreatic islets transplanted into the peritoneal cavity or thigh muscle resulted in significant improvements in multiple diabetic symptoms (including a reduction in hyperglycemia for sustained periods) in streptozotocin induced diabetic rats [26]. Even more, islet allografts reduced the severe diabetic state for sustained periods in azathioprine treated diabetic rats [26]. Rechard and Barker later increased the

number of islets transplanted to 800-1200 islets injected into the intraperitoneal cavity showing a complete reversal of diabetes for the first time [27]. As secretion of insulin to the liver represents a more physiologic approach to regulating blood glucose levels, Kemp et al., demonstrated that injection of 400-600 rat islets into the portal vein was sufficient to provide insulin independence in recipients rats [28]. To this day, infusion of islets into the portal vein remains the site for clinical islet transplantation.

While there were significant improvements in the efficacy of islet transplantation in rodent models, successful clinical islet transplantation remained elusive. This was primarily due to an inability to recover sufficient numbers of purified islets, inadequate immunosuppressive therapies, and severe complications due to transplantation of impure graft tissue (i.e. portal hypertension and disseminated intravascular coagulation) [29,30]. In 1984, Gray et al., reported an improved method of isolating islets from the human pancreas [31]. This involved injecting collagenase directly into the pancreatic duct to distend the pancreas, teasing the tissue rather than mechanical chopping, and Ficoll density gradient separation. This procedure was later improved by perfusion of collagenase via the pancreatic duct [32,33]. Further refinements in the islet isolation process followed in the subsequent years with the introduction of indraductal infusion of Liberase (a high purity blend of collagenase isoforms I and II from Clostridium histoliticum and Thermolysin from Bacillus thermoproteolyticus) [34,35], the development of a semi-automated dissociation chamber, and the utilization of a COBE for islet purification [36]. These developments allowed for the

procurement of viable islets with increased purity and enhanced the reproducibility and consistency of the procedure, facilitating the large-scale isolation and clinical applicability of the procedure [36].

Advancements in the isolation procedure combined with more effective immunosuppressive drugs (including cyclosporine and polyclonal antibody preparations) led to the first clinical islet allograft transplants in Canada in 1989 [37]. The first two transplanted patients demonstrated C-peptide secretion, however neither became insulin independent. In 1990, Scharp et al., demonstrated that insulin independence could be achieved by injecting islets from multiple donors; increasing the islet mass transplanted [38]. It was later shown by the Edmonton group that freshly isolated islets combined with cryopreserved islets from multiple donors could also lead to insulin independence, with one patient remaining insulin independent after 1.5 years of follow-up [39].

However, though insulin independence could be attained with clinical islet transplantation, majority of patients did not maintain insulin independence for a minimum of one year post-transplantation [6,38,40]. It was reported that out of the 267 islet allograft transplants done since 1990, a mere 8.2% maintained euglycemia for 1 year [41]. This was drastically changed in the year 2000 as a landmark study done by the Edmonton group demonstrated that seven out of seven patients were able to achieve and maintain normoglycemia for greater than one year post-transplantation when: *i)* islets were isolated by ductal perfusion with cold purified collagenase (Liberase) and digested in xenoprotein-free medium; *ii)* a sufficient amount of freshly isolated islets were transplanted via

percutaneous transhepatic emobolization; and *iii)* a glucocorticoid-free immunosuppressive regimen was used [41]. This protocol, later referred to as the Edmonton Protocol, utilized a mean 11, 546 islet equivalents per kilogram of body mass, as well as an immunosuppressive protocol which included the use of sirolimus, tacrolimus, and daclizumab [41]. Even more, the study demonstrated the achievement of excellent glucose control by these patients with improvements in glycemic lability and hypoglycemia [41,42]. Most importantly, it demonstrated that islet transplantation could be a realistic and effective modality of treatment for patients suffering from diabetes.

1.2.2 Current Challenges

Though islet transplantation was able to remedy a number of problems for transplanted patients, there are still a number of challenges and barriers limiting the widespread application of this therapy. Functionally, a 5 year follow-up study on patients that received an islet transplant under the Edmonton Protocol demonstrated that majority of patients required a second, and even sometimes a third transplant to achieve sustained euglycemia [41,42]. Even more, while approximately 80% showed evidence of C-peptide secretion, only about 10% of patients remained insulin independent five years after the initial transplant. Overall, the median graft survival was 15 months post-transplant, demonstrating that the islet grafts may have eventually stopped functioning or produced insufficient quantities of insulin. One counterintuitive finding was that C-peptide levels remained high for a majority of patients and did not correlate with the

percentage of patients that remained insulin independent. Possible reasons may be an increase in insulin resistance in patients, exhaustion of the islets due to high insulin demands, or a decrease in islet function due to immunological rejection or toxicity of the immunosuppressive agents [42]. Future studies will need to be done to determine the actual reasons for the decreased islet function.

From a safety perspective, while the procedure itself is minimally invasive, the immunosuppressive drugs used to prevent graft rejection result in significant toxicity and side effects, limiting the patient population for which this treatment is appropriate. Even more, patients require immunosuppression chronically as the current immunosuppressive regimes are meant to inhibit immune reactivity rather than build tolerance to graft tissue. Reported side effects of the immunosuppressive drugs included mouth ulcers, ovarian cysts, diarrhea, acne, edema, anemia, and pneumonia [42]. Thus, with the high toxicity of the current anti-rejection therapies, islet transplantation remains limited to brittle type 1 diabetic patients. If however this therapy is to be used to transplant a large number of type 1 diabetic patients, including children with type 1 diabetes, it is imperative that safer anti-rejection strategies be found so that all patients can benefit from the therapy.

From an immunological standpoint, the requirement for multiple transplants and lack of long-term graft function suggests an inadequate engraftment of the islets. The instant blood mediated inflammatory reaction (IBMIR), a thrombotic/inflammatory process, continues to be acknowledged as a major contributor toward the initial graft loss and subsequent reduction in islet

engraftment. When transplanted tissue comes in contact with host blood, tissue factor, MCP-1, and other inflammatory mediators cause a rapid activation of the coagulation and complement systems, binding of platelets, and infiltration of granulocytes and monocytes [43-45]. This results in damaging effects on islets and thrombus formation which disrupts islet morphology and integrity. The clotting also prevents proper engraftment by preventing access to blood vessels [46]. This being said, a number of methods have been looked at to prevent IBMIR including the use of low molecular weight dextran sulfate [47], heparin [44], compstatin [48], thrombin inhibitor [49], genetically modified donor pigs [50,51], and other strategies that will prevent and/or reduce IBMIR [51].

From a procedural view, islets are currently infused into the portal vein and subsequently embolize within the liver [52]. This site is advantageous because a relatively low number of islets can be used to achieve normoglycemia within this site as compared to other tested transplant sites [52]. In part, this is presumably because the liver provides a good delivery system through which the actions of insulin can be quickly spread through the rest of the body. Even more, infusion into the portal vein is a minimally invasive procedure. However there are major disadvantages with this site. For example, severe procedural complications include bleeding, thrombosis, biliary puncture, and a transient increase in aminotransferase and arteriovenous fistula can result [42]. Infusion into the portal vein was also shown to increase portal pressure [42]. Furthermore, islets are in direct contact with host blood in this site, making them more susceptible to IBMIR and subsequent losses in engrafted islet tissue [53]. Intrahepatic islets may also display

abnormalities due to exposure to high concentrations of glucagon, diabetogenic immunosuppressive drugs and their metabolites, as well as toxins from the gastrointestinal tract [54]. Biopsies are also difficult to acquire, therefore monitoring the graft becomes both difficult and more risky [54]. Thus, it is clear that a more optimal site for implantation will have to be looked at in order to increase the safety and efficacy of islet transplantation.

Lastly, as mentioned above, islet transplantation is currently reserved for a very small subset of patients for which the harmful side effects of the immunosuppressive therapies are deemed less severe than the complications of the diabetic state of these patients. As islet transplantation becomes a more available therapy for type 1 diabetics, the number of patients for which islet transplantation can be offered will greatly outweigh the availability of islets. Thus, a shortage of human donor islets will ensue and will further limit the widespread application of this therapy. One potential strategy to overcome this barrier is to use xenogeneic tissue as an alternative source of transplantable islets.

1.3 XENOTRANSPLANTATION OF PORCINE ISLETS

In 1893, the first attempt at clinical islet transplantation was undertaken by Watson-Williams and Harsant [36]. Even more interesting than the fact that transplantation of pancreatic fragments was attempted some 28 years prior to the discovery of insulin, is that Watson-Williams and Harsant used pancreatic fragments from a sheep rather than human tissue in an attempt to correct the fatal ketoacidosis that their fifteen year old patient suffered from [36].

Multiple sources of xenogeneic islet tissue suitable for transplantation have been investigated including: *i)* bovine islets [55-57], *ii)* porcine islets (including fetal, neonatal, and adult) [58-60], and *iii)* fish Brockman bodies [61-63]. However, on account of the physiologic and morphologic similarities between human and pig islets, porcine tissue seems to provide the most realistic source of xenogeneic islet tissue at present and has made the greatest strides towards being used clinically. Pigs are an attractive source for xenogeneic tissue because they breed rapidly, have large litter sizes, have a short gestation time, can be housed in pathogen-free environments, and can be genetically altered to create transgenic pigs that are safer for transplantation [50,59,64]. Even more, porcine insulin has been used clinically for the treatment of diabetes for years, demonstrating the efficacy of porcine insulin in reversing hyperglycemia in humans [65].

1.3.1 Optimal Age of Porcine Islet Donor

While there is support for using islets of porcine origin, the optimal age of pigs from which islets should be harvested is still being debated. Three main age groups have been investigated, namely adult, neonatal, and fetal.

A study conducted by Ricordi et al., demonstrated that approximately 255, 000 islets can be isolated from an adult pig using a modified version of the human islet isolation procedure [60]. The final preparation of these islets was 85-90% pure and was able to reverse hyperglycemia in nude mice. This study outlines the two major advantages of using adult islets for transplantation, those being: *i)* large numbers of islets can be isolated from a single pig which reduces the amount of

pigs necessary to transplant each patient; and *ii)* islets from adult pigs are functionally mature and are able to correct hyperglycemia without latency [66]. Functional capacity of adult porcine islets in pre-clinical non-human primates has also been shown by Hering et al., demonstrating the corrective capacity of this source of islets in a phylogenetically related species (cynomolgus macaques) [67].

However, there are significant limitations with using adult pig islets. For one, islets isolated from adult pigs are fragile and difficult to maintain in culture [60,68]. Furthermore, the quantity of islets that are isolated from an adult pancreas are dependent on the age and breed of the pig, organ quality, and the activity and lot of collagenase used, all of which result in significant variability in islet yields [65,69,70]. Adult islets are also potentially more immunogenic than neonatal pigs, furthering the need for immunosuppression [71]. Also, logistically, maintaining pigs in pathogen-free environments long-term is both difficult and costly which will again limit the applicability of this treatment. It would also become difficult to ensure pigs of a proper age are available for transplantation if you need to wait long periods of time before harvesting their pancreas.

In contrast to adult islets, neonatal and fetal islets are much more viable and less susceptible to damage during culture and cryopreservation [59]. Isolation of islets from fetal pigs has been shown to yield approximately 10,000 islet cell clusters which are able to correct hyperglycemia in alloxan induced diabetic mice within two months of the transplant [58]. Even more, fetal porcine islet-like cell clusters have been used to correct hyperglycemia in ten diabetic patients in

Uppsala, Sweden, however while C-peptide levels were detected for up to 460 days post-transplant, none of the patients achieved euglycemia [72] .

The major limitation with fetal islets is that they consistently demonstrate low insulin secretary response to glucose [59]. They are also immature and take months to correct hyperglycemia in animal models [58]. Even more, relatively low numbers of islets can be isolated from a single fetal pancreas, necessitating a greater quantity of fetal pig donors in order to isolate sufficient quantities of islets for transplanting a single recipient. For example, in the study mentioned above, 39-100 pig fetuses were required to transplant each patient.

Neonatal pigs may provide the best source of islets as they have a good insulin secretory response to glucose, are easy to isolate and maintain in culture, and have growth potential as 57% of the pancreatic isolate is believed to contain endocrine precursor cells [59,64,73]. Even more, consistent/reproducible numbers of islets can be isolated from these piglets with an approximate yield of 50,000 islet aggregates per pancreas [59]. However, reversal of hyperglycemia in diabetic nude mice required up to 8 weeks, again demonstrating the immature status of these cells [59]. This being said, neonatal porcine islets (NPI) have been shown to correct hyperglycemia in pre-clinical non-human primates (rhesus macaques) long-term, furthering the clinical applicability of this source of islets for transplantation [74]. More recently, a group in Mexico conducted a clinical trial in which they transplanted NPI and Sertoli cells (SC) into 12 diabetic patients and reported that 50% of recipients had a decreased insulin requirement for up to four years [75]. In

this study, there was no detection of any transmission of porcine endogenous retrovirus (PERV) or any other severe complications.

Aside from the lag time before complete functioning of NPI, two major disadvantages of this source are the possible high number of donor piglets required to transplant a single patient and the tremendous work that will be involved in preparing these cells for transplantation. While a more precise number will need to be determined, large animal models have demonstrated that anywhere from 14 to 70 piglets could be required to transplant a single 70 kg human patient [74,76]. Considering the amount of work and resources that are required to prepare each donor pancreas, including the multiple media changes during the 7 day culture period used for the purification of the NPI (a single pancreas requires 4 plates, each of which requires 3 media changes prior to preparation for transplantation), the large-scale application of this source of islets will be severely limited unless more efficient methods of preparing the islets are found (e.g. automating the NPI isolation procedure). In contrast, because a higher number of islets can be isolated from an adult pig pancreas and because these islets are mature, fewer adult pigs would be required to transplant a single patient (approximately 7 donor adult pigs) [67].

As mentioned previously however, long-term housing of pigs is a costly and arduous endeavor. Current regulations necessitate that donor animals be "designated pathogen-free" and are to be bred and housed as "closed herds" in a biosecure facility [77]. Animals must also be regularly assessed for pathogens and the facility must operate in compliance with "Good Manufacturing Practice"

(GMP) [77]. With these requirements, it is clear why short-term housing of animals such as neonatal piglets is more realistic and feasible than long-term housing of adult pigs. At present, there is no consensus as to which source of islets should be used, however it is apparent that islets from either neonatal or adult pigs should be used if clinical trials are to be attempted [70,78].

1.3.2 Additional Barriers Limiting Application of Clinical Porcine Islet Transplantation

Aside from the housing requirements necessary to breed donor pigs, two other obstacles currently limit the clinical application of porcine islet xenotransplantation. Firstly, transmission of pathogenic infections/viruses from porcine hosts is a major concern in terms of the safety of the donor tissue. While this problem can be partially remedied through the use of pathogen-free breeding facilities, endogenous viruses inherent within the genome of pigs, such as PERV, are difficult to screen out. Even more concerning was a study showing that PERV from a porcine cell line could infect human kidney cells (293 kidney cell line) *in vitro* [79]. To date however, there have been no known cases of transmission of PERV from recipients of porcine tissue, including patients who have received immunosuppression [73,77,80].

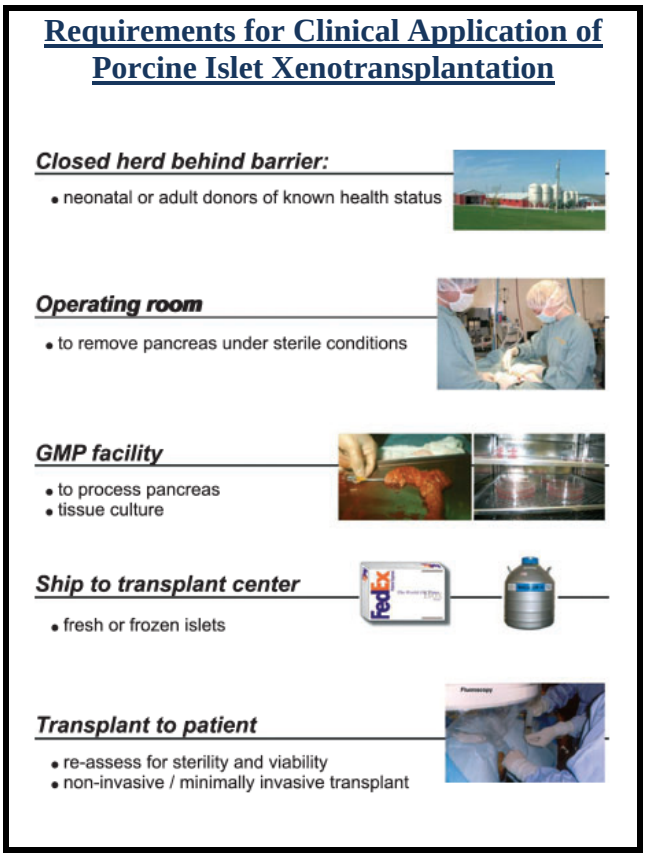

Figure 1.1: Facilities and procedural requirements for clinical islet xenotransplantation: Adapted from, Rajotte, R.V. Moving towards clinical application. Xenotransplantation 15, 113-115 (2008).

The second major obstacle limiting the clinical application of porcine islet transplantation is finding a safe and effective way to evade host immunity and the potent cellular response mounted against xenogeneic tissue. This barrier is of immense importance as further usage of harmful immunosuppressive therapies would only further limit the applicability of islet transplantation. Thus, safe and effective ways to achieve xenograft protection must be discovered in spite of the potent immune response.

1.3.3 Mechanism of Rejection of Neonatal Porcine Islets

The process of NPI xenograft rejection comprises of IBMIR, as well as innate, antibody, and cell-mediated immune responses. As indicated by the name, IBMIR occurs immediately after contact with host blood, destroying approximately 22 to 73% of the xenogeneic islet graft within the first 24 hours after transplantation [81]. Thus, IBMIR alone can sufficiently destroy islet grafts to the point of non-function in the absence of any adaptive immune response. This necessitates higher numbers of transplanted islets in order to achieve sustained insulin independence [74]. While IBMIR occurs in both allo- and xenotransplantation, the effect of IBMIR may be augmented due to the presence of natural preformed antibodies against xenogeneic antigens, exacerbating complement binding and activation. One such target to which preformed antibodies exist is galactose α(1,3)-galactose, a carbohydrate moiety found to be expressed on porcine tissue, including NPI [82]. Thus, transplantation of NPI faces the added hurdle of overcoming hyperacute rejection and IBMIR.

The contribution of innate immune cells has also been explored in xenograft rejection. Primarily eosinophils, natural killer cells, and macrophages have been shown to play a role in islet xenograft rejection [83-87]. This has been demonstrated by the infiltration of these cells within the islet grafts. However, while each may play a role, these studies have shown that depletion of macrophages has the most pronounced effect on the potency of islet xenograft rejection in comparison to the other two cell types [83,84,86].

Overall however, cell-mediated immune responses seem to be a necessary component for islet xenograft rejection. It is well established that T cell activation requires two distinct signals. The first (signal 1) is an antigen specific signal provided by the interaction between the foreign antigen (presented by an antigen presenting cell through an MHC molecule) and a T cell receptor (TCR). The second signal (signal 2) or co-stimulatory signal, in and of itself is unable to stimulate T cells; however it augments and amplifies activation. This is accomplished via decreasing the threshold of activation, increasing expression of adhesion molecules, and preventing anergy. Overall, this process leads to the production of cytokines, as well as proliferation and differentiation of effector cells, all of which lead to a full blown immune reaction [88,89]. In the absence of this second signal, T cells are believed to enter a state of anergy or unresponsiveness.

T cell mediated immune responses play a critical role in the rejection of islet xenografts, however the mechanism or pathway through which this is achieved is dependent on the phylogenetic disparity between the host and donor [90]. In the direct pathway of antigen recognition, host T cells can be directly activated by antigen presentation from donor MHC molecules, whereas in the indirect pathway of recognition, host antigen presenting cells (APC) process and present antigen to T cells, resulting in immune responsiveness and graft rejection.

Whereas direct antigen recognition is dominant in allograft rejection [91], it appears that there is a larger role for the indirect pathway of recognition in genetically disparate (discordant) xenograft models. In a study done by Rayat and Gill, it was shown that the significance or contribution of indirect recognition

increased as a function of the disparity between the donor and host [90]. In this study, C57BL/6 lymphocytes could respond directly to allogeneic BALB/c or phylogenetically related (concordant) rat stimulator cells in a mixed lymphocyte reaction. In contrast, no reactivity was seen when C57BL/6 lymphocytes were cultured with neonatal porcine stimulator cells, suggesting that antigen processing is necessary for activation of phylogenetically disparate lymphocytes. *In vivo*, C2D mice (MHC class II deficient) receiving BALB/c islet allografts rejected their grafts at a comparable rate as wild-type mice. In contrast, survival of Wistar-Furth rat islets in C2D mice was significantly prolonged, while NPI xenografts uniformly survived long-term, demonstrating the progressively increased importance of $CD4^+$ T cells and the indirect pathway of recognition as the phylogenetic disparity between host and donor is increased.

Thus, as $CD4^+$ T lymphocytes and indirect antigen presentation are mandatory for discordant islet xenograft rejection, therapies that specifically target this cell type/pathway without producing toxic side effects will need to be found. More specifically, short-term therapies that can induce tolerance to the NPI grafts will be important in furthering the clinical application of islet xenotransplantation for the treatment of type 1 DM. One potential method of achieving this is to use monoclonal antibody (mAb) therapies that can directly interfere with important pathways in T cell activation and function such as anti-LFA-1, anti-CD154, or anti-CD45RB mAb.

1.4 MONOCLONAL ANTIBODY THERAPIES

1.4.1 Anti-LFA-1 mAb

Adhesion molecules have clearly demonstrated their role in cell-cell interaction and in various aspects of host defense. This is highlighted by the extreme susceptibility toward infection and severe immunological complications seen in patients with defects in these proteins [92,93]. Leukocyte function associated antigen-1 (LFA-1), a major adhesion molecule expressed on a wide variety of hematopoiec cells including macrophages, monocytes, granulocytes, NK cells, and most heavily on T and B cells, is a β2 integrin with a heterodimer structure consisting of a CD11a α chain and a CD18 β chain [94-96]. Its primary targets are ICAM-1 and ICAM-2 on endothelial cells, ICAM-1 and ICAM-3 on APC, and JAM-1 found at tight junctions of endothelial and epithelial cells [97,98]. There are four potentially important roles of this integrin which make it an attractive therapeutic target for evading immune rejection.

Firstly, LFA-1 binding to ICAM-1 arrests the rolling of leukocytes at the endothelial surface [99]. Initially, passing leukocytes bind L-selectin causing them to roll along the endothelial surface. In order for these leukocytes to cross through the endothelial surface however, leukocytes must be arrested so extravasation can take place. LFA-1 plays a key role in this, binding firmly to the rolling leukocytes and arresting their movement [99].

Secondly, LFA-1 acts as a pro-migratory molecule, playing an important role in transendothelial migration and trafficking of lymphocytes [100,101]. With ICAM-1 laying a path through which leukocytes pass, binding of this molecule to LFA-1 essentially leads the leukocyte through the endothelial surface and to the

site of inflammation. Thus, blocking of this interaction potentially counters the ability of leukocytes to reach their targets as seen in LFA-1 deficient mice [102].

Thirdly, LFA-1 plays a critical role at the interface between APC and T cells [103]. As the TCR binds to its specific antigen presenting MHC molecule, sustained periods of engagement are necessary in order to adequately activate T cells to proliferate and carry out more complex functions. This cannot be achieved merely through engagement of the TCR to the MHC molecule due to: *i)* steric hindrance from other surface molecules; *ii)* low affinity between TCRs and MHC molecules; and *iii)* low numbers of antigen complexes available for binding [104]. Thus, additional adhesion molecules are required in order to sustain this interface. This is supported as a study done by Abraham et al., showed that a 10,000 fold increase in TCR antigen presentation required to induce proliferation in the presence of LFA-1/ICAM-1 interaction was still insufficient to induce proliferation in the absence of this interaction [105].

It has been shown that upon initial contact between the T cell and APC, clusters of LFA-1/ICAM-1 form in the central region of this interface with TCR binding more towards the periphery. Eventually, the engaged TCRs all move to the center of this interface with a ring like structure of adhesion molecules surrounding it, holding it firmly in place [104]. This process allows for firm adhesion between the T cell and the APC via LFA-1 and also serves to maximize TCR/MHC binding due to the clustering of these molecules, further optimizing activation [104].

Lastly, LFA-1 has been implicated in providing additional activation signals or co-stimulation to T cells. As mentioned above LFA-1/ICAM-1 interaction seemed critical for the activation and proliferative response of T cells, as increasing the TCR ligand presentation was still unable to evoke a sufficient response in the absence of this interaction. Further, antigen presentation by transfectants expressing MHC and ICAM-1 in the absence of other co-stimulatory molecules have been shown to increase IL-2 production and proliferation in naïve cells, whereas transfectants expressing MHC alone did not [106]. LFA-1/ICAM-1 interaction also transiently increases IL-2 gene expression in T_H1 clones [106]. Support for the co-stimulatory functions of LFA-1 also comes from studies demonstrating that LFA-1/ICAM-1 binding can lead to increased inositol phospholipid hydrolysis, appearance of the hyperphosphorylated p23 form of the TCR ζ chain, sustained intracellular calcium levels, and an increase in cytoplasmic calcium levels [103]. Two distinct types of signaling have been recognized upon engagement of LFA-1 on T cells with its corresponding ligands on APC. First is an inside-out signal which increases the avidity of the integrin. This signaling involves cytoplasmic proteins such as talin and cytohesin 1 [107]. The second signal is an outside-in signal believed to affect intracellular signaling [107]. This process is believed to involve the transcription factor Jun activation domain binding protein or JAB-1 (shown to be involved in the cell cycle as well as regulation of the cytoskeleton), as well as cytohesin 1 [107-109]. It has also recently been shown that engagement of LFA-1 with ICAM-1 in conjunction with TCR signaling greatly enhances Ras activation, an important regulator of T cell

development, proliferation, and apoptotic cell death [110,111]. Overall, the net effect of these processes is a decrease in the threshold of T cell activation [112].

In a mixed lymphocyte reaction (*in vitro* proliferation assay), anti-LFA-1 mAb effectively inhibited lymphocyte proliferation in response to both allo- [112] and xenoantigen [113]. Anti-LFA-1 mAb has demonstrated efficacy in multiple transplant models including cardiac, tracheal, and islet graft models [113-117]. Specifically in an islet allograft model, the extent of the protection induced by anti-LFA-1 mAb was highly dependent upon the strain of the recipient mouse, as 89% of CBA recipients receiving BALB/c islets remained normoglycemic for greater than 100 days post transplant, compared to 39% in C57BL/6 mice [116]. Interestingly, anti-LFA-1 mAb treatment in this model also led to tolerance which was adoptively transferred to immune deficient mice. Further, anti-donor reactivity was preserved in tolerant immune cells as assessed by proliferative and cytotoxic assays demonstrating the mechanism of anti-LFA-1 mAb induced protection was not clonal deletion or anergy [116]. Transfusion of donor spleen cells was also used to rule out the possibility that ignorance was the basis for protection [116]. Transplantation of allogeneic islets into autoimmune non-obese diabetic (NOD) mice demonstrated that anti-LFA-1 mAb alone is not sufficient to prolong islet graft survival, however did significantly prolong allograft survival when combined with anti-CD154 mAb [112]. Interestingly, targeting LFA-1 and/or its ligand ICAM-1 has been shown to both prevent and reverse the occurrence of diabetes in both chemically induced and autoimmune diabetes models [118-120].

Anti-LFA-1 mAb has also been shown to be effective in islet xenograft models, both concordant and discordant. In a rat to mouse islet transplant model, Tredget et al., showed that short-term administration of anti-LFA-1 mAb prevented rat islet xenograft rejection for >100 days in 27 out of 28 recipients [113]. In contrast, in a discordant xenograft model where NPI were transplanted into diabetic C57BL/6 mice, only 7/15 mice achieved normoglycemia with only 6 of the 15 mice achieving long-term graft survival when treated with anti-LFA-1 mAb [117]. This protection however, was greatly enhanced with the addition of anti-CD154 mAb to the anti-rejection regimen as 12/14 mice receiving NPI achieved long-term graft survival with this combination of mAbs [117].

The current humanized anti-LFA-1 treatment Efalizumab (targets the CD11a α chain) however, has failed to provide consistent effectiveness [103]. Even more, higher doses were shown to increase the risk of developing lymphoproliferative disease [121]. Thus, though anti-LFA-1 mAb therapy has the potential to be a useful therapy, methods to improve its effectiveness with low doses will need to be found. In the treatment of psoriasis however, Efalizumab was well tolerated, with majority of patients experiencing an improvement with the treatment. Acute side effects were mild and included headaches, nausea, myalgia, chills, and fever [122]. Currently, Efalizumab is being tested as an anti-rejection therapy for islet transplant recipients.

1.4.2 Anti-CD154 mAb

It has been established that antigen-receptor ligation in the absence of co-stimulatory signals results in T cell unresponsiveness. As such, these co-stimulatory interactions have become major therapeutic targets. One such co-stimulatory pathway involves the interaction of CD40 and CD154 (also known as CD40L). The interaction between these molecules has been shown to have an immense impact on the activation of host immunity, having a multi-faceted role in the activation of both APC and T cells.

CD40 is a transmembrane glycoprotein expressed on all APC (B-cells, dendritic cells, macrophages, among others), on activated T-cells, hematopoeic progenitor cells, as well as non-hematopoeic cells such as endothelial cells [123]. Its ligand, CD154 is found on activated T and B cells, activated platelets [124], and on a number of other cells during inflammation such as peripheral blood mononuclear cells [125,126].

When CD40 is bound by its ligand, a signal transduction pathway is triggered leading to the transcription of target genes [126]. Although the complete mechanism has not yet been defined, it is believed that following binding, a trimeric structure is formed. CD40 does not have kinase activity alone but is able to recruit secondary messengers such as janus kinase or members of the TNF receptor associated factor (TRAF) family [123]. TRAFs in particular bind to a number of protein kinases leading to the activation of MAPK and NF-κB which ultimately increases gene expression. TRAFs have also been shown to activate gene expression directly as they possess DNA binding motifs capable of regulating transcription [123,126].

CD40/CD154 interaction seems to provide signals to both APC and T cells directly. Binding to CD40 is critical for the maturation of APC, promoting antigen presentation and an increased expression of co-stimulatory (i.e. CD80 and CD86) and adhesion (i.e. ICAM-1) molecules [123,127,128]. Even more, APC have been shown to increase cytokine and chemokine production after CD40/CD154 interaction [123,127]. For example, dendritic cells have been shown to secrete increased levels of TNF-α, IL-12, IL-8, all of which are known to be pro-inflammatory [129].

The binding of CD40 has been shown to be extremely important in B cell differentiation and maturation. Binding of CD40 to CD154 leads to an up-regulation of co-stimulatory molecules such as CD80 and CD86 [123]. This interaction also leads to clonal expansion as well as maturation of B cells into long-lived plasma cells [130]. CD154-CD40 binding also plays a fundamental role in class switching of antibody isotypes [126]. This function is quite clear in Hyper IgM Syndrome in which mutations lead to a defective CD154 gene. This disease is characterized by normal or high levels of IgM, but low levels of other isotypes (IgG, IgE, and IgA) demonstrating an inability to class switch [130]. Hyper IgM Syndrome has also been shown to have detrimental effects on T cells and T cell priming, which ultimately results in a severe immune deficiency [130]. Further, by up-regulating co-stimulatory signals on APC, up-regulating IL-2 receptors on T cells, and through the release of various cytokines, the binding of CD40 with its ligand clearly plays a key role in the priming and proliferation of both cytotoxic T

cells and helper T cells [123,131-134]. Thus, CD40/CD40L interaction is also important in T cell response [135].

Anti-CD154 mAb therapy has been shown to be an effective form of therapy in a number of transplant models including cardiac, skin, bone marrow, and skin transplantation, especially when combined with a donor specific transfusion of splenocytes [125,136]. In non-human primate islet allograft models, humanized anti-CD154 (hu5c8) mAb therapy has demonstrated significant efficacy in preventing graft rejection. In a baboon model it was shown that induction therapy with anti-CD154 mAb was sufficient in allowing pancreactomized baboons receiving islet allografts to achieve normoglycemia for at least 30 days [137]. Even more, anti-CD154 mAb treatment administered at the onset of rejection was sufficient in reversing rejection in these patients, allowing long-term graft survival [137]. This was also seen in rhesus monkeys, as six out of six monkeys transplanted with allogeneic islets achieved long-term graft function with only humanized anti-CD154 mAb therapy. This model did however require monthly maintenance therapy with anti-CD154 mAb [138]. Delving more into the mechanism of protection induced by anti-CD154 mAb, Beilke et al., presented the novel finding that anti-CD154 mAb mediated protection was dependent on NK cells [139]. Here it was shown that the absence of NK cells and not CD8 or NKT cells, completely abrogates the protective effects of anti-CD154 mAb, and that NK cells promote this protection through a perforin-dependent mechanism [139].

Anti-CD154 mAb has also been shown to be partially effective in autoimmune transplant models. DR-BB autoimmune diabetic rats treated with

hamster anti-rat CD154 mAb significantly prolonged graft survival with no recurrence of diabetes for up to 461 days post-transplant [140]. Allotransplantation of C57BL/6 islets into spontaneously diabetic NOD female mice showed significant graft prolongation with anti-CD154 mAb therapy as mice treated with isotype control had a median graft survival of 10 days whereas higher dose anti-CD154 mAb prolonged graft survival to a median of 46 days [141].

Interference of the CD40/CD154 pathway has also been shown to have potent effects on xenograft rejection in both concordant and discordant xenograft models. Mai et al., have shown that short-term anti-CD154 mAb treatment increased mean graft survival to >120 days in mice receiving either concordant rat islets or discordant human islets [142]. This same group later showed that prolonged graft survival was attained in mice that were re-transplanted with discordant HLA-mismatched human islets in the absence of additional therapy [143]. In contrast, anti-CD154 mAb has demonstrated only a partial or moderate efficacy in preventing NPI xenograft rejection when administered as a monotherapy, with only ~ 40% of mice achieving normoglycemia [117,144]. This protection was greatly enhanced when anti-CD154 mAb is combined with either a donor specific transfusion of spleen cells [144] or other mAbs [117]. Most recently it has been shown that addition of anti-CD154 mAb to the established immunosuppressive regimes effectively prolonged neonatal and adult pig islet xenografts in non-human primates [67,74].

Though anti-CD154 mAb is a promising therapy, especially when combined with other treatments, it has fallen out of favor due to evidence of

thromboembolic complications in both non-human primates and humans (phase-1 clinical trials) [88]. This may in part be due to CD154 function on activated platelets. Administration of heparin during this therapy did reduce thromboembolic events [136]. Overall however, the above information shows that targeting this receptor-ligand pair or pathway can greatly reduce the immune responses involved in graft rejection and may possibly lead to tolerance induction via blockade of imperative co-stimulatory signals.

1.4.3 Anti-CD45RB mAb

The balance between kinase and phosphatase activity is a major determinant of many cellular processes. The same is true in lymphocyte activation as demonstrated by the function of CD45, also known as leukocyte common antigen. This family of transmembrane glycoproteins is expressed on all nucleated hematopoietic cells and their precursors, thus serving as a major cell surface marker for leukocytes [145,146]. CD45 is a type 1 protein tyrosine phosphatase with a cytoplasmic region that is highly conserved between species and is believed to play a critical role in antigen-receptor signaling [147]. The extracellular region of this molecule exists in multiple forms based on the alternative splicing of three exons designated as A, B, and C in their protein structure. Thus, while CD45 is expressed on all nucleated hematopoietic cells, different isotopes of this molecule are restricted to particular cells at different developmental stages [148].

The importance of CD45 in immunological processes is clearly demonstrated by CD45 knockout models and CD45 deficient cell lines which

show defects in the activation and maturation of T and B lymphocytes [147,149]. Even more, altered expression or genetic mutations in CD45 result in severe-combined immunodeficiency (SCID) in humans [150,151], while polymorphisms in this molecule have been associated with autoimmunity and malignant diseases [148]. CD45 has also been shown to be involved in cytokine receptor signaling albeit negatively via dephosphorylation of JAKs [152].

The primary mechanism for the positive regulatory role of CD45 is believed to be through dephosphorylation of inhibitory regions of Src kinases (p56lck and p56fyn) which are involved in relaying the initial signal generated through the antigen receptor and in phosphorylation of downstream targets leading to activation [145]. Essentially, TCR engagement leads to phosphorylation of p56lck and p56fyn, however the phosphotyrosine residue located at the COOH-terminal of these Src family kinases is bound to the SH2 domain of the same molecule in its phosphorylated form. As such, CD45 acts as a positive regulator of T and B cell activation by dephosphorylating this inhibitory region allowing kinase activity to continue [145].

In vitro, targeting CD45RB rather than CD45 or CD45RA was effective in inhibiting T cell proliferation in a mixed lymphocyte reaction [153]. These cells also showed a decreased expression of IL-2 receptors as determined via flow cytometry. Inhibition of alloreactivity has also been shown in various allogeneic transplant models including cardiac, renal, and islet graft models [154-156].

The protection induced by anti-CD45RB mAb in islet allograft models shows a shift in isotype expression from $CD45RB^{hi}$ to $CD45RB^{lo}$ and

consequently an increase in IL-4 and IL-10 [156]. Upon second challenge with an islet graft from the same donor strain, 75% of protected mice remained normoglycemic for >50 days, suggesting that the therapy induced a state of tolerance [156]. Interestingly, CTLA-4, a known co-inhibitory molecule, is constitutively expressed on a portion of these $CD45RB^{lo}$ cells and anti-CTLA-4 mAb administered concomitantly with anti-CD45RB mAb therapy abrogates the protective effects of the treatment [157]. Even more, cyclosporine A, a calcineurin inhibitor, was used to prevent up-regulation of CTLA-4 with anti-CD45RB mAb treatment which also inhibited the protective effects of the mAb. This demonstrates that up-regulation of CTLA-4 may play a crucial role in anti-CD45RB mAb mediated protection [157]. Anti-CD45RB mAb has also shown some efficacy in the prevention of islet allograft rejection in an autoimmune model of diabetes (the NOD mouse), however none of the treated mice achieved long-term graft survival [158].

In an islet xenograft model, rat islets transplanted into mice had significantly prolonged survival after monotherapy with anti-CD45RB, although none of the mice achieved graft survival >100 days post-transplant [159]. In another xenograft model, fetal porcine pancreas fragments were transplanted into mice [160]. The mechanism of protection proposed for the prolongation of fetal pancreatic tissue with anti-CD45RB mAb monotherapy was in part explained by a decrease in T cell priming and trafficking. This was concluded based on evidence that there was a decrease in both T_H1 and T_H2 cytokine production, a decrease in immune cell infiltration within the graft, and a decrease in the T cell pool within lymph

nodes of recipient mice, attributable to a decreased expression of adhesion molecules LFA-1 and CD62L [160]. Lastly, in a study done by Rayat and Gill, anti-CD45RB mAb showed only a moderate efficacy in preventing NPI xenograft rejection in diabetic C57BL/6 mice, as only three of the eight mice achieved normoglycemia, with two achieving long-term graft survival [117]. Overall, these studies suggest that a multitude of mechanisms may be involved in the protection observed with anti-CD45RB mAb therapy, however when used as a monotherapy, anti-CD45RB mAb is only partially effective in preventing islet xenograft rejection.

1.5 IMMUNOPROTECTION

There are particular areas in the body known to provide immune protection to local tissues. These sites have been deemed immune privileged as tissue grafted into these sites have extended survival compared to other conventional sites [161]. These sites include the brain, the anterior chamber of the eye, the placenta, and the testes [162]. From an evolutionary perspective the protection within these sites is quite intuitive, as damage due to inflammation within these areas would result in significant deterioration of daily functioning and/or reproductive capacity. The testis is particularly interesting as it not only prevents inflammatory reactions to avoid bystander killing, but also prevents immune rejection of post-meiotic germ cells which display unique surface antigens that can elicit an immune response [163]. This capacity to confer protection makes the testicular environment particular interesting for transplantation.

Originally it was thought that immune privilege within this site was a result of segregation from the immune system, however it has now become clear that processes outside of general immune ignorance play an important role in the maintenance of this immunoprotective testicular environment. For example, the testis is able to protect autoantigenic material in spite of the high degree of lymphatic drainage in this organ. Also, there are points especially at the rete testes, where gaps exist, permitting the passage of immune cells. This negates the possibility that ignorance alone is responsible for the immunoprivileged status of the testes [163,164].

1.5.1 Immune Privilege in the Testis

The testis contains seminiferous tubules surrounded by a layer of myoid cells. The extratubular or interstitial compartment contains Leydig cells, blood vessels, fibroblasts, macrophages, and lymph [10,165]. Within the seminiferous tubules of the testes are the SC and germ cells. It is within these seminiferous tubules that developing germ cells undergo spermatogenesis, expressing surface antigens that would be rejected if placed in another part of the body [163]. As mentioned above, it was initially believed that this escape from rejection occurred as a result of being segregated from immune cells via the blood-testes barrier. This barrier is formed by adjacent SC or "nurse cells," which create a continuous membrane-like structure via tight junctions. This continuous structure therefore creates two separate compartments. The basal side contains spermatogonia or pre-meiotic germ cells which are in contact with material from the vascular system;

whereas the adluminal compartment containing meiotic and post-meiotic germ cells (expressing a plethora of surface antigens) is segregated from these materials. Though in part this may play a role, it has been shown that there are points permitting infiltration of immune cells [163]. Even more, foreign tissues placed in the testes can produce Ag-specific systemic immunity, thus showing an interaction with the immune system [166]. It was later shown that testicular fluids contained immunosuppressive compounds that have the potential to reduce immunity [167,168].

Via selective disruption of different cell types within the testis, it was shown that it is SC that are the essential component in maintaining testicular immune privilege. The necessity for germ cells and spermatogenesis in the establishment of immunoprotection was analyzed by transplanting parathyroid allografts within a SC only testes (created by low dose irradiation) or testis that had been anchored into the abdomen (rendered cryptorchid), respectively [169]. Here it was shown that immunoprotection was still maintained in the absence of germ cells and spermatogenesis. The role of Leydig cells was later explored by destroying this cell type with ethane dimethanesulfonate [170]. This experiment confirmed that Leydig cells are not essential for testicular immune privilege. The effect of steroid production was later determined by disruption of steroidogenesis via leuprolide or hypophysectomy [171]. Intratesticular islet grafts were still protected under these conditions demonstrating that local steroidogenesis, including testosterone and progesterone production, are not necessary for immunoprotection within the testes. Thus, it would appear that understanding

immune privilege in the testis lies in an understanding of the factors secreted by SC.

1.6 SERTOLI CELLS

SC were first discovered in 1865 by Enrico Sertoli [172]. Besides their role in the blood-testis barrier, SC are also involved in: *i)* the movement of germ cells and the release of sperm; *ii)* phagocytosis of degenerated germ cells; *iii)* the secretion of various nutrients and growth factors; and *iv)* spermatogenesis [165,172].

In terms of their immunosuppressive properties, SC have been shown to produce a variety of products capable of reducing inflammatory responsiveness. *In vitro*, SC secretions have been shown to significantly decrease proliferation of lymphocytes and decrease IL-2 production, arresting these cells within the G1 phase of the cell cycle [168]. Interestingly, addition of exogenous IL-2 failed to overcome the inhibitory effect of SC conditioned media on the proliferation of lymphocytes [167]. SC conditioned media also significantly reduces cytotoxic T lymphocyte mediated killing and DNA fragmentation of target cells by inhibiting granzyme B [173]. SC have also demonstrated an ability to evade natural antibody-mediated lysis *in vitro*, as incubation with human serum and complement was unable to effectively destroy SC [174]. Here it was shown that SC secrete molecules that can inhibit complement mediated destruction and the formation of the membrane attack complex (MAC) [174]. Four major products that have been implicated in SC immunosuppressive activity are FasL, TGF-β, clusterin, and serine protease inhibitors.

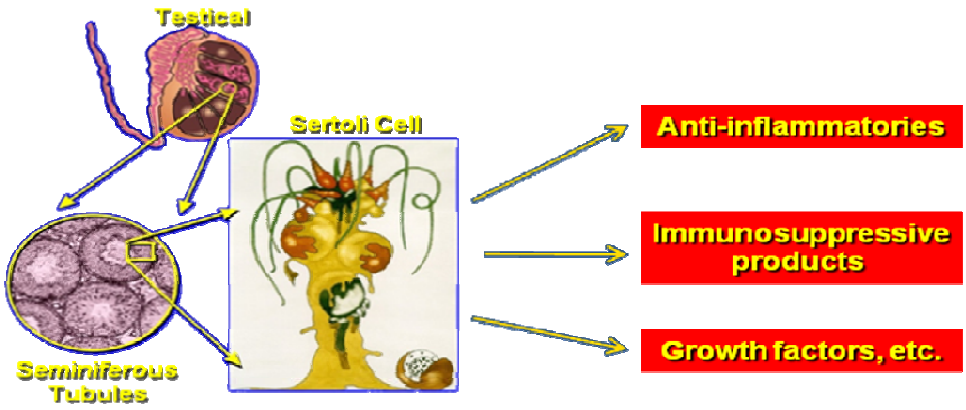

Figure 1.2: Immunoprotection from SC. Figure kindly provided by Dr. Rajotte.

1.6.1 Fas/FasL

Fas, also known as CD95 or Apo-1, is a type 1 transmembrane protein belonging to the tumor necrosis factor family. It is found on many cell types including hepatocytes, neutrophils, and activated T and B cells, and has been shown to have immune modulating functions [175,176]. Fas binding to its ligand FasL induces apoptosis in Fas expressing cells [176]. There is support for the idea that Fas interaction with FasL may be important in regulating lymphocyte numbers through peripheral deletion, as lymphocytes transiently express both Fas and FasL [176,177]. Even more, mutations in Fas/FasL lead to lymphoproliferative disorders, as well as autoimmunity in both mice and humans [178,179].

Immune privileged sites including the anterior chamber of the eye and the testis have both been shown to secrete Fas and thus came about the idea that expression of this molecule could lead to immune privilege. This idea was supported as mouse testicular allografts expressing FasL survived, whereas testicular grafts that did not express FasL were rapidly rejected [180]. Even more,

syngeneic myoblasts engineered to produce FasL protected islet allografts from rejection when placed under the kidney capsule [181]. Though this seemed like the solution to rejection, a number of conflicting findings have been found.

In a study done by Kang et al., islets were genetically engineered to express FasL using recombinant adenoviral vectors [182]. Here, it was found that the engineered islet grafts underwent an accelerated rejection compared to the control. This rejection was shown to be T and B cell independent, as a similar outcome occurred when transplanted into immune deficient SCID mice. Histological examination revealed heavy neutrophilic infiltrate [182]. Similar results were found in a study done by Korbutt et al., in which FasL expression was correlated with rejection when syngeneic islets and SC were transplanted into NOD mice [183]. In this study, it was shown that FasL expression was lower in mice that had prolonged graft survival, whereas those mice that rejected their graft had higher levels of FasL and an increased neutrophilic infiltrate [183]. Even more, anti-FasL antibody was shown to enhance survival of islet grafts in mice transplanted with islets and SC, suggesting that FasL has a detrimental effect on the islets [184]. Thus, it would appear that other factors must be involved in SC mediated immunoprotection.

1.6.2 TGF-β

Transforming growth factor β (TGF-β) is a cytokine secreted by SC that has emerged as a key regulator of immune responses. TGF-β is well known for its immune modulating and anti-inflammatory functions, inhibiting both T cell

proliferation [185] and IL-2 production [186]. TGF-β also plays a major role in T regulatory cell mediated suppression, immune tolerance, and in the induction of foxp3$^+$ T regulatory cells from CD4$^+$CD25$^-$ precursors [187-189]. As a result, TGF-β has been proposed as a critical feature in SC mediated protection of co-transplanted islets [184].

This was supported by a study done by Suarez-Pinzon et al., which showed that the protection induced by co-transplanting syngeneic islets with SC in NOD mice was completely abrogated when anti-TGF-β was administered [184]. In this model, anti-TGF-β was also shown to promote IFN-γ and reduce IL-4 production, suggesting that TGF-β may play a role in immune modulation, shifting the immune response from a T_H1 mediated to a T_H2 mediated immune response. Transgenic islets expressing TGF-β have also been shown to protect NOD mice from diabetes [190]. Thus, it appears TGF-β is a necessary component in the immunoprotective effects of SC.

In addition to its suppressive role on the immune system, TGF-β may also reduce the immunogenicity of the grafted tissue. Carel et al., have shown that culturing rat xenogeneic islets with recombinant TGF-β for 7 days significantly prolonged survival of the transplanted islets in the absence of any other therapies (rat to mouse transplant model) [191]. Therefore, TGF-β may protect islet grafts via multiple mechanisms including direct inhibition of inflammation and immune cell activation, enhancing the development of regulatory cells, and modulation of grafted tissue.

1.6.3 Other Factors

Cytotoxic T lymphocytes (CTL) can play an important role in graft rejection, especially in allotransplantation. One major mechanism through which grafts are rejected via CTL is through the release of granzyme B which induces DNA fragmentation and apoptosis in target cells. Using SC conditioned media, Sipione et al., showed that SC secrete a serpin (serine proteinase inhibitor) that binds irreversibly to granzyme B, thus inhibiting its destructive effects. This serpin is called serpina3n, and may be another important mechanism through which SC prevent graft rejection [173].

Clusterin is another molecule secreted by SC that has anti-inflammatory properties [10], and is also capable of inhibiting complement mediated destruction of target tissue by inhibiting MAC formation [10,174,192]. Therefore, SC may be able to inhibit humoral mediated rejection as well. Overall, these different secreted products likely work together in creating a proper milieu for preventing immune reactivity and rejection.

1.6.4 Expression of Co-inhibitory Molecules and Generation of T Regulatory Cells

Investigation of the expression of co-inhibitory molecules on SC has provided further insights on the potential mechanisms through which SC can prevent immune reactivity. A recent study has suggested that SC can act as non-professional tolerogenic APC as they can transmit negative signals to $CD8^+$ T cells and induce T regulatory cells [193]. In this study, mouse SC responded to IFN-

γ by expressing both MHC class II and B7-H1 (also known as PD-L1) [193]. Ligation of B7-H1 was shown to transmit inhibitory signals, as proliferation of $CD8^+$ T cells was shunted when they were co-cultured with SC. Addition of neutralizing anti-B7-H1 to co-cultured $CD8^+$ T cells and SC enhanced proliferation of the $CD8^+$ T cells. $CD4^+$ T cell proliferation was also decreased *in vitro* when co-cultured with SC, however this was independent of B7-H1 as blockade of this molecule did not diminish the suppression. Instead, it was shown that $CD4^+CD25^+foxp3^+$ T regulatory cells were significantly increased when $CD4^+$ T cells were co-cultured with IFN-γ stimulated SC. This finding is supported by a recent *in vivo* study by Nasr et al., where intratesticular islet grafts resulted in a reduction of $CD8^+$ memory T cells and an increase in T regulatory cells compared to islets transplanted under the kidney capsule [166].

1.6.5 Effect of SC on Islet Function and Maturation

In addition to the immunomodulatory and immunoprotective properties of SC, a body of evidence has suggested that SC may provide additional benefits to the islets themselves. *In vitro* culturing of islets with SC has been shown to have mitogenic effects on the islets as shown by Brd-U labeling [194]. Even more, islets co-cultured with SC have a higher response to glucose stimulation [194,195]. This together suggests that SC may facilitate the maturation, expansion, and functioning/responsiveness of islets. As SC are responsible for providing trophic support and nutrients for germ cells in the native testis, it is intuitive that they may provide the same type of support to islets via the production of various growth

factors such as insulin growth factor-1 (IGF-1), insulin growth factor-2 (IGF-2), and epidermal growth factor (EGF) [194]. This is supported by a study done by Basta et al., in which neonatal porcine pancreatic endocrine precursor cells showed enhanced differentiation into insulin secreting endocrine cells when cultured with SC conditioned media [196]. As mentioned previously, one of the major problems or limitations with clinical islet transplantation at present is the diminishment of islet function over time. Thus, co-transplantation of islets with SC, in addition to preventing islet graft rejection, may augment the health and expansion of islets, sustaining proper function.

1.6.6 Co-transplantation of Islets and Sertoli Cells

Various tissues including human breast sarcoma, skin, parathyroid fragments, and insulinoma tissue have shown prolonged survival when placed in the testicular environment, although to varying degrees [10]. In terms of islets, a study done by Ferguson and Scothornet showed that islet allografts transplanted into guinea pigs showed extended survival when placed in the testes [197]. Though this showed that islets can survive for extended periods in the testes (up to 11 weeks post-transplant), it did not show their ability to correct hyperglycemia. Selawry and Whittington later demonstrated that intratesticular grafts that were left in their normal scrotal position were unable to correct hyperglycemia, however, were able to when intratesticular grafts were anchored into the abdominal cavity [198]. More specifically with NPI, Gores et al., showed that long-term NPI xenograft survival could be achieved in non-immunosuppressed (no

additional immunosuppressive drugs were necessary) beagles when NPI were grafted into the intra-abdominally placed testis [199]. This study showed that NPI can be protected and can correct hyperglycemia in a large animal model. However, because intratesticular grafting would only be relevant for male patients and because these patients would become infertile when their testes were anchored into the abdomen, intratesticular islet grafts are not clinically relevant for islet transplantation.

Therefore, in order to test whether neonatal porcine SC could provide protection in a physiologically relevant site, Dufour et al., transplanted neonatal pig SC under the kidney capsule of Lewis rats [200]. In 66% of the transplanted rats, xenogeneic SC survived for > 90 days post-transplant without any immune therapy. Thus, SC were able to protect themselves outside the testicular environment in this model [200].

To determine if SC could confer this protection to islet grafts in the absence of any other immunosuppression, Korbutt et al., co-transplanted 2000 allogeneic islets and $11x10^6$ allogeneic SC under the kidney capsule of Wistar-Furth rats. Without any immunosuppressive therapy, 5/5 recipients maintained graft survival for >95 days [201]. Korbutt et al., then tested the effectiveness of co-transplantation of syngeneic islets and SC in an autoimmune model of diabetes, the NOD mouse [183]. It was shown that co-transplantation of SC with islets prolonged graft survival and that there was a dose-dependent relationship between the number of SC implanted with islets and the longevity of graft survival. For example, NOD mice receiving islets alone had a median graft survival of 11 days,

those that received islets with $2x10^6$ SC had a median graft survival of 28 days, and those receiving islets and $4x10^6$ SC had a median graft survival of 32 days post-transplantation. Interestingly, islets transplanted with $8x10^6$ SC reduced graft survival to 8 days which suggests that co-transplanting excess numbers of SC can have deleterious effects on islets and that there is an optimal ratio of islets and SC that should be transplanted [183]. Thus, though the effect was not as pronounced as in the previous allogeneic model, co-transplantation showed effectiveness in protecting syngeneic islet grafts from autoimmune destruction.

In xenogeneic models however, it has been consistently shown that co-transplantation of islets and SC alone is insufficient in preventing islet xenograft rejection. In a concordant xenograft model Lewis rat islets and BALB/c SC were transplanted under the kidney capsule of BALB/c mice. Here, neither co-transplantation of islets with SC alone nor the administration of anti-lymphocyte serum with islets alone significantly prolonged graft survival. However, when islets were transplanted with SC and a single injection of anti-lymphocyte serum (ALS) was administered, graft survival bolstered from 10.9±0.8 days (mean graft survival in islet alone group) to 64.9±8.1 days, with 6 of the 25 recipients having graft survival for >100 days [202].

In a discordant fish to mouse islet xenograft model, it was shown that SC were able to significantly prolong islet xenograft survival when islets and SC were co-encapsulated [62]. Here, co-transplantation of islets and SC was not done in the absence of an alginate microcapsule and so it is not possible to determine the contribution of the SC in the absence of the alginate barrier. However, even with

both treatment strategies, the mean graft survival was only 46 days, suggesting that SC alone would likely be unable to prevent graft rejection. The requirement for an additional strategy to be combined with SC is further supported as NPI xenografts co-transplanted with SC were rapidly rejected when transplanted into diabetic rats or non-human primates [203,204]. This again shows that SC alone are not sufficient to prevent islet xenograft rejection when used as a monotherapy.

In summary, co-transplantation of islets and SC has the potential to be an effective treatment strategy; however, for sustained long-term islet graft survival in autoimmune and xenograft models an additional strategy will be required. If this strategy proves to be safe and effective, the use of SC may further the clinical applicability and wide spread application of NPI xenotransplantation into type 1 diabetic patients, as it may decrease or remove the requirement for toxic immunosuppressive therapies by enhancing the efficacy of potentially safer alternative therapies (i.e. mAb therapies).

1.7 OBJECTIVES AND OUTLINE

The data presented thus far suggests that neither SC alone nor monotherapy of mAb provides complete protection to NPI xenografts. Thus, the objective of this thesis is to determine if co-transplanting NPI with SC combined with short-term administration of anti-LFA-1, anti-CD154, or anti-CD45RB will prevent islet xenograft rejection. We hypothesize that this combination of treatment modalities will result in long-term NPI xenograft survival.

This hypothesis is tested in Chapter 2, as C57BL/6 mice were transplanted with either NPI alone or NPI with SC. These mice then received one of the three mAb therapies or were left untreated. After 100 days post-transplantation, the morphology of the grafted tissue and immune cell infiltrate was analyzed using immunohistochemistry to see if there were differences based on treatment. As regulatory T cells have been implicated in the protection induced by mAb therapies and SC, graft tissue was also stained for foxp3 (marker for T regulatory cells) to determine if T regulatory cells were present within the graft site. Relative production of anti-porcine antibodies was also determined so as to provide a more quantitative assessment of the differences in antigen specific reactivity that was mounted against the grafted tissue based on treatment.

Overall, this study was designed to address two important barriers limiting the widespread application of islet transplantation. Firstly, to remedy the shortage of donor tissue, the use of NPI has been proposed as an alternative source of transplantable islets. Therefore in our study, we wanted to determine the potential of the mAb therapies and SC in preventing NPI xenograft rejection and to determine whether long-term survival and function of these islets could be achieved. This in essence would further the clinical applicability of using NPI for the treatment of type 1 diabetes and would alleviate the demand for islet tissue. Secondly, the chronic administration of harmful immunosuppressive anti-rejection therapies has severely limited the widespread application of islet transplantation. To address this issue, we are investigating the potential of short-term mAb therapies combined with immunoprotective SC to provide long-term

protection to the islet grafts, removing the requirement for chronic immunosuppression. Thus overall, the goal of our study is to investigate a potential strategy to prevent NPI xenograft rejection so as to facilitate the widespread application of islet transplantation.

1.8 REFERENCES

1. Kasper, D.L. & Harrison, T.R. *Harrison's principles of internal medicine*, (McGraw-Hill, Medical Pub. Division, New York, 2005).
2. Pickup, J.C. & Williams, G. *Textbook of diabetes*, (Blackwell Science, Malden, Mass., 2003).
3. Kaufman, F.R. & American Diabetes Association. *Medical management of type 1 diabetes*, (American Diabetes Association, Alexandria, Va., 2008).
4. Diagnosis and classification of diabetes mellitus. *Diabetes Care* **27 Suppl 1**, S5-S10 (2004).
5. The prevalence and costs of diabetes. (Canadian Diabetes Association, 2005-2009).
6. Merani, S. & Shapiro, A.M. Current status of pancreatic islet transplantation. *Clin Sci (Lond)* **110**, 611-625 (2006).
7. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* **26 Suppl 1**, S5-20 (2003).
8. Ichii, H. & Ricordi, C. Current status of islet cell transplantation. *J Hepatobiliary Pancreat Surg* **16**, 101-112 (2009).
9. Lieberman, M., Marks, A.D., Smith, C.M. & Marks, D.B. *Marks' essential medical biochemistry*, (Lippincott Williams & Wilkins, Philadelphia, 2007).
10. Dufour, J.M., Rajotte, R.V., Korbutt, G.S. & Emerich, D.F. Harnessing the immunomodulatory properties of Sertoli cells to enable xenotransplantation in type I diabetes. *Immunol Invest* **32**, 275-297 (2003).
11. Filippi, C. & von Herrath, M. How viral infections affect the autoimmune process leading to type 1 diabetes. *Cell Immunol* **233**, 125-132 (2005).
12. Schrezenmeir, J. & Jagla, A. Milk and diabetes. *J Am Coll Nutr* **19**, 176S-190S (2000).
13. Danescu, L.G., Levy, S. & Levy, J. Vitamin D and diabetes mellitus. *Endocrine* **35**, 11-17 (2009).
14. Luong, K., Nguyen, L.T. & Nguyen, D.N. The role of vitamin D in protecting type 1 diabetes mellitus. *Diabetes Metab Res Rev* **21**, 338-346 (2005).
15. Mathieu, C. & Badenhoop, K. Vitamin D and type 1 diabetes mellitus: state of the art. *Trends Endocrinol Metab* **16**, 261-266 (2005).
16. Matthews, D. *Diabetes*, (Oxford University Press, Oxford ; New York, 2008).
17. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. *N Engl J Med* **329**, 977-986 (1993).
18. von Mering J, M.O. *Arch Exp Pathol Pharmakol* **26**, 371 (1889).
19. Minkowski, O. Weitere Mitteilungen uber den diabetes mellitus nach extirpation des pankreas. *Berl Klin Wochenschr* **29**, 90-93.

20. Williams, P. Notes on diabetes treated with extract and by grafts of sheep's pancreas. *British Medical Journal* **2**, 1303-1304 (1894).
21. Hellerstrom, C. A method for the microdissection of intact pancreatic islets of mammals. *Acta Endocrinol* **45**, 122-132 (1964).
22. Moskalewski, S. Isolation and Culture of the Islets of Langerhans of the Guinea Pig. *Gen Comp Endocrinol* **44**, 342-353 (1965).
23. Lacy, P.E. & Kostianovsky, M. Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* **16**, 35-39 (1967).
24. Lindall, A., Steffes, M. & Sorenson, R. Immunoassayable insulin content of subcellular fractions of rat islets. *Endocrinology* **85**, 218-223 (1969).
25. Younoszai R, S.R., Lindall A. Homotransplantation of isolated pancreatic islets. *Diabetes* **19 (Abstract)**, 406 (1970).
26. Ballinger, W.F. & Lacy, P.E. Transplantation of intact pancreatic islets in rats. *Surgery* **72**, 175-186 (1972).
27. Reckard, C.R., Ziegler, M.M. & Barker, C.F. Physiological and immunological consequences of transplanting isolated pancreatic islets. *Surgery* **74**, 91-99 (1973).
28. Kemp, C.B., Knight, M.J., Scharp, D.W., Lacy, P.E. & Ballinger, W.F. Transplantation of isolated pancreatic islets into the portal vein of diabetic rats. *Nature* **244**, 447 (1973).
29. Nason, R.W., Rajotte, R.V. & Warnock, G.L. Pancreatic islet cell transplantation: past, present and future. *Diabetes Res* **7**, 1-11 (1988).
30. Warnock, G.L., and Rajotte, R.V. Pancreatic Islet Cell Transplantation. *Canadian Family of Physicians* **38**, 1655-1660 (1992).
31. Gray, D.W., McShane, P., Grant, A. & Morris, P.J. A method for isolation of islets of Langerhans from the human pancreas. *Diabetes* **33**, 1055-1061 (1984).
32. Warnock, G.L., *et al.* Studies of the isolation and viability of human islets of Langerhans. *Transplantation* **45**, 957-963 (1988).
33. Warnock, G.L., *et al.* Viable purified islets of Langerhans from collagenase-perfused human pancreas. *Diabetes* **38 Suppl 1**, 136-139 (1989).
34. Linetsky, E., *et al.* Comparison of collagenase type P and Liberase during human islet isolation using the automated method. *Transplant Proc* **27**, 3264 (1995).
35. Linetsky, E., *et al.* Improved human islet isolation using a new enzyme blend, liberase. *Diabetes* **46**, 1120-1123 (1997).
36. Shapiro, A.M.J. & Shaw, J.A.M. *Islet transplantation and beta cell replacement therapy*, (Informa Healthcare, New York, 2007).
37. Warnock, G.L., *et al.* Continued function of pancreatic islets after transplantation in type I diabetes. *Lancet* **2**, 570-572 (1989).
38. Scharp, D.W., *et al.* Insulin independence after islet transplantation into type I diabetic patient. *Diabetes* **39**, 515-518 (1990).
39. Warnock, G.L., Kneteman, N.M., Ryan, E.A., Rabinovitch, A. & Rajotte, R.V. Long-term follow-up after transplantation of insulin-producing

pancreatic islets into patients with type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* **35**, 89-95 (1992).
40. Ricordi, C. & Strom, T.B. Clinical islet transplantation: advances and immunological challenges. *Nat Rev Immunol* **4**, 259-268 (2004).
41. Shapiro, A.M.*, et al.* Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* **343**, 230-238 (2000).
42. Ryan, E.A.*, et al.* Five-year follow-up after clinical islet transplantation. *Diabetes* **54**, 2060-2069 (2005).
43. Moberg, L.*, et al.* Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. *Lancet* **360**, 2039-2045 (2002).
44. Cabric, S.*, et al.* Islet surface heparinization prevents the instant blood-mediated inflammatory reaction in islet transplantation. *Diabetes* **56**, 2008-2015 (2007).
45. Bennet, W.*, et al.* Damage to porcine islets of Langerhans after exposure to human blood in vitro, or after intraportal transplantation to cynomologus monkeys: protective effects of sCR1 and heparin. *Transplantation* **69**, 711-719 (2000).
46. Johansson, H. Acta Universitatis Upsaliensis Uppsala (2007).
47. Johansson, H.*, et al.* Low molecular weight dextran sulfate: a strong candidate drug to block IBMIR in clinical islet transplantation. *Am J Transplant* **6**, 305-312 (2006).
48. Tjernberg, J., Ekdahl, K.N., Lambris, J.D., Korsgren, O. & Nilsson, B. Acute antibody-mediated complement activation mediates lysis of pancreatic islets cells and may cause tissue loss in clinical islet transplantation. *Transplantation* **85**, 1193-1199 (2008).
49. Ozmen, L.*, et al.* Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: possible application of the thrombin inhibitor melagatran in clinical islet transplantation. *Diabetes* **51**, 1779-1784 (2002).
50. d'Apice, A.J. & Cowan, P.J. Gene-modified pigs. *Xenotransplantation* **15**, 87-90 (2008).
51. van der Windt, D.J., Bottino, R., Casu, A., Campanile, N. & Cooper, D.K. Rapid loss of intraportally transplanted islets: an overview of pathophysiology and preventive strategies. *Xenotransplantation* **14**, 288-297 (2007).
52. Merani, S., Toso, C., Emamaullee, J. & Shapiro, A.M. Optimal implantation site for pancreatic islet transplantation. *Br J Surg* **95**, 1449-1461 (2008).
53. Bennet, W., Groth, C.G., Larsson, R., Nilsson, B. & Korsgren, O. Isolated human islets trigger an instant blood mediated inflammatory reaction: implications for intraportal islet transplantation as a treatment for patients with type 1 diabetes. *Ups J Med Sci* **105**, 125-133 (2000).
54. Contreras, J.L. Extrahepatic transplant sites for islet xenotransplantation. *Xenotransplantation* **15**, 99-101 (2008).

55. Figliuzzi, M., *et al.* Subcutaneous xenotransplantation of bovine pancreatic islets. *Biomaterials* **26**, 5640-5647 (2005).
56. Marchetti, P., *et al.* Massive isolation, morphological and functional characterization, and xenotransplantation of bovine pancreatic islets. *Diabetes* **44**, 375-381 (1995).
57. Lanza, R.P., Kuhtreiber, W.M., Ecker, D., Staruk, J.E. & Chick, W.L. Xenotransplantation of porcine and bovine islets without immunosuppression using uncoated alginate microspheres. *Transplantation* **59**, 1377-1384 (1995).
58. Korsgren, O., Jansson, L., Eizirik, D. & Andersson, A. Functional and morphological differentiation of fetal porcine islet-like cell clusters after transplantation into nude mice. *Diabetologia* **34**, 379-386 (1991).
59. Korbutt, G.S., *et al.* Large scale isolation, growth, and function of porcine neonatal islet cells. *J Clin Invest* **97**, 2119-2129 (1996).
60. Ricordi, C., *et al.* Isolation of the elusive pig islet. *Surgery* **107**, 688-694 (1990).
61. Wright, J.R., Jr., Pohajdak, B., Xu, B.Y. & Leventhal, J.R. Piscine islet xenotransplantation. *ILAR J* **45**, 314-323 (2004).
62. Yang, H. & Wright, J.R., Jr. Co-encapsulation of Sertoli enriched testicular cell fractions further prolongs fish-to-mouse islet xenograft survival. *Transplantation* **67**, 815-820 (1999).
63. Wright, J.R., Jr., Polvi, S. & MacLean, H. Experimental transplantation with principal islets of teleost fish (Brockmann bodies). Long-term function of tilapia islet tissue in diabetic nude mice. *Diabetes* **41**, 1528-1532 (1992).
64. Binette, T.M., Dufour, J.M. & Korbutt, G.S. In vitro maturation of neonatal porcine islets: a novel model for the study of islet development and xenotransplantation. *Ann N Y Acad Sci* **944**, 47-61 (2001).
65. Socci, C., *et al.* Selection of donors significantly improves pig islet isolation yield. *Horm Metab Res Suppl* **25**, 32-34 (1990).
66. Bottino, R., *et al.* Isolation outcome and functional characteristics of young and adult pig pancreatic islets for transplantation studies. *Xenotransplantation* **14**, 74-82 (2007).
67. Hering, B.J., *et al.* Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. *Nat Med* **12**, 301-303 (2006).
68. van Deijnen, J.H., Hulstaert, C.E., Wolters, G.H. & van Schilfgaarde, R. Significance of the peri-insular extracellular matrix for islet isolation from the pancreas of rat, dog, pig, and man. *Cell Tissue Res* **267**, 139-146 (1992).
69. Kirchhof, N., Hering, B.J., Geiss, V., Federlin, K. & Bretzel, R.G. Evidence for breed-dependent differences in porcine islets of Langerhans. *Transplant Proc* **26**, 616-617 (1994).
70. Korbutt, G.S. What type of islets should be used? *Xenotransplantation* **15**, 81-82 (2008).

71. Bloch, K., *et al.* Neonatal pig islets induce a lower T-cell response than adult pig islets in IDDM patients. *Transplantation* **67**, 748-752 (1999).
72. Groth, C.G., *et al.* Transplantation of porcine fetal pancreas to diabetic patients. *Lancet* **344**, 1402-1404 (1994).
73. Rayat, G.R., Rajotte, R.V. & Korbutt, G.S. Potential application of neonatal porcine islets as treatment for type 1 diabetes: a review. *Ann N Y Acad Sci* **875**, 175-188 (1999).
74. Cardona, K., *et al.* Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat Med* **12**, 304-306 (2006).
75. Valdes-Gonzalez, R.A., *et al.* Xenotransplantation of porcine neonatal islets of Langerhans and Sertoli cells: a 4-year study. *Eur J Endocrinol* **153**, 419-427 (2005).
76. Kin, T., Korbutt, G.S., Kobayashi, T., Dufour, J.M. & Rajotte, R.V. Reversal of diabetes in pancreatectomized pigs after transplantation of neonatal porcine islets. *Diabetes* **54**, 1032-1039 (2005).
77. Schuurman, H.J. Regulatory aspects of pig-to-human islet transplantation. *Xenotransplantation* **15**, 116-120 (2008).
78. Rajotte, R.V. Moving towards clinical application. *Xenotransplantation* **15**, 113-115 (2008).
79. Patience, C., Takeuchi, Y. & Weiss, R.A. Infection of human cells by an endogenous retrovirus of pigs. *Nat Med* **3**, 282-286 (1997).
80. MacKenzie, D.A., Hullett, D.A. & Sollinger, H.W. Xenogeneic transplantation of porcine islets: an overview. *Transplantation* **76**, 887-891 (2003).
81. Nilsson, B. The instant blood-mediated inflammatory reaction in xenogeneic islet transplantation. *Xenotransplantation* **15**, 96-98 (2008).
82. Rayat, G.R., Rajotte, R.V., Elliott, J.F. & Korbutt, G.S. Expression of Gal alpha(1,3)gal on neonatal porcine islet beta-cells and susceptibility to human antibody/complement lysis. *Diabetes* **47**, 1406-1411 (1998).
83. Simeonovic, C.J., *et al.* Eosinophils are not required for the rejection of neovascularized fetal pig proislet xenografts in mice. *J Immunol* **158**, 2490-2499 (1997).
84. Wu, G.S., *et al.* Role of macrophages and natural killer cells in the rejection of pig islet xenografts in mice. *Transplant Proc* **32**, 1069 (2000).
85. Karlsson-Parra, A., *et al.* Xenograft rejection of porcine islet-like cell clusters in normal and natural killer cell-depleted mice. *Transplantation* **61**, 1313-1320 (1996).
86. Wu, G., *et al.* Pig islet xenograft rejection is markedly delayed in macrophage-depleted mice: a study in streptozotocin diabetic animals. *Xenotransplantation* **7**, 214-220 (2000).
87. Omer, A., *et al.* Macrophage depletion improves survival of porcine neonatal pancreatic cell clusters contained in alginate macrocapsules transplanted into rats. *Xenotransplantation* **10**, 240-251 (2003).
88. Clarkson, M.R. & Sayegh, M.H. T-cell costimulatory pathways in allograft rejection and tolerance. *Transplantation* **80**, 555-563 (2005).

89. Sayegh, M.H. & Turka, L.A. The role of T-cell costimulatory activation pathways in transplant rejection. *N Engl J Med* **338**, 1813-1821 (1998).
90. Rayat, G.R., *et al.* The degree of phylogenetic disparity of islet grafts dictates the reliance on indirect CD4 T-cell antigen recognition for rejection. *Diabetes* **52**, 1433-1440 (2003).
91. Wolf, L.A., Coulombe, M. & Gill, R.G. Donor antigen-presenting cell-independent rejection of islet xenografts. *Transplantation* **60**, 1164-1170 (1995).
92. Van Epps, D.E., Potter, J., Vachula, M., Smith, C.W. & Anderson, D.C. Suppression of human lymphocyte chemotaxis and transendothelial migration by anti-LFA-1 antibody. *J Immunol* **143**, 3207-3210 (1989).
93. Anderson, D.C., *et al.* The severe and moderate phenotypes of heritable Mac-1, LFA-1 deficiency: their quantitative definition and relation to leukocyte dysfunction and clinical features. *J Infect Dis* **152**, 668-689 (1985).
94. Milicevic, N.M., Nohroudi, K., Milicevic, Z. & Westermann, J. Blood lymphocytes, monocytes and NK cells modulate their expression of CD44, ICAM-1, LFA-1 and MHC class II after arrival into lymphoid organs. *Immunol Invest* **33**, 439-452 (2004).
95. Kurzinger, K., *et al.* A novel lymphocyte function-associated antigen (LFA-1): cellular distribution, quantitative expression, and structure. *J Immunol* **127**, 596-602 (1981).
96. Dougherty, G.J. & Hogg, N. The role of monocyte lymphocyte function-associated antigen 1 (LFA-1) in accessory cell function. *Eur J Immunol* **17**, 943-947 (1987).
97. Go, S., *et al.* Anti-LFA-1 antibody postpones T-cell receptor triggering while preserving generation of regulatory T cells in T-cell receptor anti-HY transgenic mice. *Transplantation* **82**, 119-126 (2006).
98. Ostermann, G., Weber, K.S., Zernecke, A., Schroder, A. & Weber, C. JAM-1 is a ligand of the beta(2) integrin LFA-1 involved in transendothelial migration of leukocytes. *Nat Immunol* **3**, 151-158 (2002).
99. Warnock, R.A., Askari, S., Butcher, E.C. & von Andrian, U.H. Molecular mechanisms of lymphocyte homing to peripheral lymph nodes. *J Exp Med* **187**, 205-216 (1998).
100. Kavanaugh, A.F., Lightfoot, E., Lipsky, P.E. & Oppenheimer-Marks, N. Role of CD11/CD18 in adhesion and transendothelial migration of T cells. Analysis utilizing CD18-deficient T cell clones. *J Immunol* **146**, 4149-4156 (1991).
101. Hamann, A., *et al.* Evidence for an accessory role of LFA-1 in lymphocyte-high endothelium interaction during homing. *J Immunol* **140**, 693-699 (1988).
102. Andrew, D.P., *et al.* Transendothelial migration and trafficking of leukocytes in LFA-1-deficient mice. *Eur J Immunol* **28**, 1959-1969 (1998).
103. Nicolls, M.R. & Gill, R.G. LFA-1 (CD11a) as a therapeutic target. *Am J Transplant* **6**, 27-36 (2006).

104. Grakoui, A., *et al.* The immunological synapse: a molecular machine controlling T cell activation. *Science* **285**, 221-227 (1999).
105. Abraham, C., Griffith, J. & Miller, J. The dependence for leukocyte function-associated antigen-1/ICAM-1 interactions in T cell activation cannot be overcome by expression of high density TCR ligand. *J Immunol* **162**, 4399-4405 (1999).
106. Zuckerman, L.A., Pullen, L. & Miller, J. Functional consequences of costimulation by ICAM-1 on IL-2 gene expression and T cell activation. *J Immunol* **160**, 3259-3268 (1998).
107. Dustin, M.L., Bivona, T.G. & Philips, M.R. Membranes as messengers in T cell adhesion signaling. *Nat Immunol* **5**, 363-372 (2004).
108. Bianchi, E., *et al.* Integrin LFA-1 interacts with the transcriptional co-activator JAB1 to modulate AP-1 activity. *Nature* **404**, 617-621 (2000).
109. Perez, O.D., *et al.* Leukocyte functional antigen 1 lowers T cell activation thresholds and signaling through cytohesin-1 and Jun-activating binding protein 1. *Nat Immunol* **4**, 1083-1092 (2003).
110. Mor, A., *et al.* The lymphocyte function-associated antigen-1 receptor costimulates plasma membrane Ras via phospholipase D2. *Nat Cell Biol* **9**, 713-719 (2007).
111. Gomez, J., Martinez, A.C., Fernandez, B., Garcia, A. & Rebollo, A. Critical role of Ras in the proliferation and prevention of apoptosis mediated by IL-2. *J Immunol* **157**, 2272-2281 (1996).
112. Berney, T., *et al.* The effect of simultaneous CD154 and LFA-1 blockade on the survival of allogeneic islet grafts in nonobese diabetic mice. *Transplantation* **76**, 1669-1674 (2003).
113. Tredget, E.B., Arefanian, H., Gill, R.G., Rajotte, R.V. & Rayat, G.R. Monotherapy with anti-LFA-1 monoclonal antibody promotes long-term survival of rat islet xenografts. *Cell Transplant* **17**, 599-608 (2008).
114. Poston, R.S., *et al.* Effects of humanized monoclonal antibody to rhesus CD11a in rhesus monkey cardiac allograft recipients. *Transplantation* **69**, 2005-2013 (2000).
115. Morikawa, M., Brazelton, T.R., Berry, G.J. & Morris, R.E. Prolonged inhibition of obliterative airway disease in murine tracheal allografts by brief treatment with anti-leukocyte function-associated antigen-1 (CD11a) monoclonal antibody. *Transplantation* **71**, 1616-1621 (2001).
116. Nicolls, M.R., Coulombe, M., Yang, H., Bolwerk, A. & Gill, R.G. Anti-LFA-1 therapy induces long-term islet allograft acceptance in the absence of IFN-gamma or IL-4. *J Immunol* **164**, 3627-3634 (2000).
117. Rayat, G.R. & Gill, R.G. Indefinite survival of neonatal porcine islet xenografts by simultaneous targeting of LFA-1 and CD154 or CD45RB. *Diabetes* **54**, 443-451 (2005).
118. Herold, K.C., Vezys, V., Gage, A. & Montag, A.G. Prevention of autoimmune diabetes by treatment with anti-LFA-1 and anti-ICAM-1 monoclonal antibodies. *Cell Immunol* **157**, 489-500 (1994).

119. Hasegawa, Y., *et al.* Prevention of autoimmune insulin-dependent diabetes in non-obese diabetic mice by anti-LFA-1 and anti-ICAM-1 mAb. *Int Immunol* **6**, 831-838 (1994).
120. Bertry-Coussot, L., *et al.* Long-term reversal of established autoimmunity upon transient blockade of the LFA-1/intercellular adhesion molecule-1 pathway. *J Immunol* **168**, 3641-3648 (2002).
121. Vincenti, F. What's in the pipeline? New immunosuppressive drugs in transplantation. *Am J Transplant* **2**, 898-903 (2002).
122. Lebwohl, M., *et al.* A novel targeted T-cell modulator, efalizumab, for plaque psoriasis. *N Engl J Med* **349**, 2004-2013 (2003).
123. Xu, Y. & Song, G. The role of CD40-CD154 interaction in cell immunoregulation. *J Biomed Sci* **11**, 426-438 (2004).
124. Henn, V., *et al.* CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* **391**, 591-594 (1998).
125. Kirk, A.D., Blair, P.J., Tadaki, D.K., Xu, H. & Harlan, D.M. The role of CD154 in organ transplant rejection and acceptance. *Philos Trans R Soc Lond B Biol Sci* **356**, 691-702 (2001).
126. Quezada, S.A., Jarvinen, L.Z., Lind, E.F. & Noelle, R.J. CD40/CD154 interactions at the interface of tolerance and immunity. *Annu Rev Immunol* **22**, 307-328 (2004).
127. Kiener, P.A., *et al.* Stimulation of CD40 with purified soluble gp39 induces proinflammatory responses in human monocytes. *J Immunol* **155**, 4917-4925 (1995).
128. Caux, C., *et al.* Activation of human dendritic cells through CD40 cross-linking. *J Exp Med* **180**, 1263-1272 (1994).
129. Bleharski, J.R., Niazi, K.R., Sieling, P.A., Cheng, G. & Modlin, R.L. Signaling lymphocytic activation molecule is expressed on CD40 ligand-activated dendritic cells and directly augments production of inflammatory cytokines. *J Immunol* **167**, 3174-3181 (2001).
130. Lougaris, V., Plebani, A., Badolato, R. & Ferrari, S. Hyper immunoglobulin M syndrome due to CD40 deficiency: clinical, molecular, and immunological features. *Immunological Reviews* **203**, 48-66 (2005).
131. Grewal, I.S., *et al.* Requirement for CD40 ligand in costimulation induction, T cell activation, and experimental allergic encephalomyelitis. *Science* **273**, 1864-1867 (1996).
132. Scaria, A., *et al.* Antibody to CD40 ligand inhibits both humoral and cellular immune responses to adenoviral vectors and facilitates repeated administration to mouse airway. *Gene Ther* **4**, 611-617 (1997).
133. Stuber, E., Strober, W. & Neurath, M. Blocking the CD40L-CD40 interaction in vivo specifically prevents the priming of T helper 1 cells through the inhibition of interleukin 12 secretion. *J Exp Med* **183**, 693-698 (1996).
134. Yang, Y. & Wilson, J.M. CD40 ligand-dependent T cell activation: requirement of B7-CD28 signaling through CD40. *Science* **273**, 1862-1864 (1996).

135. Grewal, I.S., Xu, J. & Flavell, R.A. Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. *Nature* **378**, 617-620 (1995).
136. Yamada & Sayegh, M.H. The CD154-CD40 costimulatory pathway in transplantation. *Transplantation* **73**, S36-39 (2002).
137. Kenyon, N.S.*, et al.* Long-term survival and function of intrahepatic islet allografts in baboons treated with humanized anti-CD154. *Diabetes* **48**, 1473-1481 (1999).
138. Kenyon, N.S.*, et al.* Long-term survival and function of intrahepatic islet allografts in rhesus monkeys treated with humanized anti-CD154. *Proc Natl Acad Sci U S A* **96**, 8132-8137 (1999).
139. Beilke, J.N., Kuhl, N.R., Van Kaer, L. & Gill, R.G. NK cells promote islet allograft tolerance via a perforin-dependent mechanism. *Nat Med* **11**, 1059-1065 (2005).
140. Kover, K.L., Geng, Z., Hess, D.M., Benjamin, C.D. & Moore, W.V. Anti-CD154 (CD40L) prevents recurrence of diabetes in islet isografts in the DR-BB rat. *Diabetes* **49**, 1666-1670 (2000).
141. Molano, R.D.*, et al.* Prolonged islet graft survival in NOD mice by blockade of the CD40-CD154 pathway of T-cell costimulation. *Diabetes* **50**, 270-276 (2001).
142. Mai, G.*, et al.* Role of CD40-CD154 pathway in the rejection of concordant and discordant xenogeneic islets. *Transplant Proc* **37**, 460-462 (2005).
143. Bucher, P.*, et al.* Transplantation of discordant xenogeneic islets using repeated therapy with anti-CD154. *Transplantation* **79**, 1545-1552 (2005).
144. Appel, M.C.*, et al.* Prolonged survival of neonatal porcine islet xenografts in mice treated with a donor-specific transfusion and anti-CD154 antibody. *Transplantation* **77**, 1341-1349 (2004).
145. Penninger, J.M., Irie-Sasaki, J., Sasaki, T. & Oliveira-dos-Santos, A.J. CD45: new jobs for an old acquaintance. *Nat Immunol* **2**, 389-396 (2001).
146. Altin, J.G. & Sloan, E.K. The role of CD45 and CD45-associated molecules in T cell activation. *Immunol Cell Biol* **75**, 430-445 (1997).
147. Trowbridge, I.S. & Thomas, M.L. CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. *Annu Rev Immunol* **12**, 85-116 (1994).
148. Hermiston, M.L., Zikherman, J. & Zhu, J.W. CD45, CD148, and Lyp/Pep: critical phosphatases regulating Src family kinase signaling networks in immune cells. *Immunol Rev* **228**, 288-311 (2009).
149. Byth, K.F.*, et al.* CD45-null transgenic mice reveal a positive regulatory role for CD45 in early thymocyte development, in the selection of CD4+CD8+ thymocytes, and B cell maturation. *J Exp Med* **183**, 1707-1718 (1996).
150. Cale, C.M.*, et al.* Severe combined immunodeficiency with abnormalities in expression of the common leucocyte antigen, CD45. *Arch Dis Child* **76**, 163-164 (1997).

151. Kung, C., *et al.* Mutations in the tyrosine phosphatase CD45 gene in a child with severe combined immunodeficiency disease. *Nat Med* **6**, 343-345 (2000).
152. Irie-Sasaki, J., *et al.* CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling. *Nature* **409**, 349-354 (2001).
153. Lazarovits, A.I., Poppema, S., White, M.J. & Karsh, J. Inhibition of alloreactivity in vitro by monoclonal antibodies directed against restricted isoforms of the leukocyte-common antigen (CD45). *Transplantation* **54**, 724-729 (1992).
154. Zhang, Z., *et al.* Prevention of heart allograft and kidney xenograft rejection by monoclonal antibody to CD45RB. *Transplant Proc* **29**, 1253 (1997).
155. Lazarovits, A.I., *et al.* Prevention and reversal of renal allograft rejection by antibody against CD45RB. *Nature* **380**, 717-720 (1996).
156. Basadonna, G.P., *et al.* Antibody-mediated targeting of CD45 isoforms: a novel immunotherapeutic strategy. *Proc Natl Acad Sci U S A* **95**, 3821-3826 (1998).
157. Fecteau, S., *et al.* CTLA-4 up-regulation plays a role in tolerance mediated by CD45. *Nat Immunol* **2**, 58-63 (2001).
158. Molano, R.D., *et al.* Prolonged survival of allogeneic islet grafts in NOD mice treated with a combination of anti-CD45RB and anti-CD154 antibodies. *Transplant Proc* **33**, 248-249 (2001).
159. Visser, L., *et al.* Prolonged survival of rat islet xenografts in mice after CD45RB monotherapy. *Transplantation* **77**, 386-391 (2004).
160. Sutherland, R.M., *et al.* Anti-CD45RB antibody deters xenograft rejection by modulating T cell priming and homing. *Int Immunol* **14**, 953-962 (2002).
161. Streilein, J.W. Unraveling immune privilege. *Science* **270**, 1158-1159 (1995).
162. Cobbold, S.P., *et al.* Immune privilege induced by regulatory T cells in transplantation tolerance. *Immunol Rev* **213**, 239-255 (2006).
163. Filippini, A., *et al.* Control and impairment of immune privilege in the testis and in semen. *Hum Reprod Update* **7**, 444-449 (2001).
164. Head, J.R., Neaves, W.B. & Billingham, R.E. Immune privilege in the testis. I. Basic parameters of allograft survival. *Transplantation* **36**, 423-431 (1983).
165. Jegou, B. The Sertoli cell in vivo and in vitro. *Cell Biol Toxicol* **8**, 49-54 (1992).
166. Nasr, I.W., *et al.* Testicular immune privilege promotes transplantation tolerance by altering the balance between memory and regulatory T cells. *J Immunol* **174**, 6161-6168 (2005).
167. Selawry, H.P., Kotb, M., Herrod, H.G. & Lu, Z.N. Production of a factor, or factors, suppressing IL-2 production and T cell proliferation by Sertoli cell-enriched preparations. A potential role for islet transplantation in an immunologically privileged site. *Transplantation* **52**, 846-850 (1991).

168. De Cesaris, P., *et al.* Immunosuppressive molecules produced by Sertoli cells cultured in vitro: biological effects on lymphocytes. *Biochem Biophys Res Commun* **186**, 1639-1646 (1992).
169. Whitmore, W.F., 3rd, Karsh, L. & Gittes, R.F. The role of germinal epithelium and spermatogenesis in the privileged survival of intratesticular grafts. *J Urol* **134**, 782-786 (1985).
170. Cameron, D.F., Whittington, K., Schultz, R.E. & Selawry, H.P. Successful islet/abdominal testis transplantation does not require Leydig cells. *Transplantation* **50**, 649-653 (1990).
171. Selawry, H.P. & Whittington, K.B. Prolonged intratesticular islet allograft survival is not dependent on local steroidogenesis. *Horm Metab Res* **20**, 562-565 (1988).
172. Griswold, M.D. The central role of Sertoli cells in spermatogenesis. *Semin Cell Dev Biol* **9**, 411-416 (1998).
173. Sipione, S., *et al.* Identification of a novel human granzyme B inhibitor secreted by cultured sertoli cells. *J Immunol* **177**, 5051-5058 (2006).
174. Dufour, J.M., Hamilton, M., Rajotte, R.V. & Korbutt, G.S. Neonatal porcine Sertoli cells inhibit human natural antibody-mediated lysis. *Biol Reprod* **72**, 1224-1231 (2005).
175. Nagata, S. Fas and Fas ligand: a death factor and its receptor. *Adv Immunol* **57**, 129-144 (1994).
176. Lettau, M., Paulsen, M., Kabelitz, D. & Janssen, O. Storage, expression and function of Fas ligand, the key death factor of immune cells. *Curr Med Chem* **15**, 1684-1696 (2008).
177. Green, D.R. & Ware, C.F. Fas-ligand: privilege and peril. *Proc Natl Acad Sci U S A* **94**, 5986-5990 (1997).
178. Rieux-Laucat, F., *et al.* Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science* **268**, 1347-1349 (1995).
179. Nagata, S. & Suda, T. Fas and Fas ligand: lpr and gld mutations. *Immunol Today* **16**, 39-43 (1995).
180. Takeda, Y., *et al.* Protection of islet allografts transplanted together with Fas ligand expressing testicular allografts. *Diabetologia* **41**, 315-321 (1998).
181. Lau, H.T., Yu, M., Fontana, A. & Stoeckert, C.J., Jr. Prevention of islet allograft rejection with engineered myoblasts expressing FasL in mice. *Science* **273**, 109-112 (1996).
182. Kang, S.M., *et al.* Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction. *Nat Med* **3**, 738-743 (1997).
183. Korbutt, G.S., Suarez-Pinzon, W.L., Power, R.F., Rajotte, R.V. & Rabinovitch, A. Testicular Sertoli cells exert both protective and destructive effects on syngeneic islet grafts in non-obese diabetic mice. *Diabetologia* **43**, 474-480 (2000).

184. Suarez-Pinzon, W., *et al.* Testicular sertoli cells protect islet beta-cells from autoimmune destruction in NOD mice by a transforming growth factor-beta1-dependent mechanism. *Diabetes* **49**, 1810-1818 (2000).
185. Kehrl, J.H., *et al.* Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J Exp Med* **163**, 1037-1050 (1986).
186. Das, L. & Levine, A.D. TGF-beta inhibits IL-2 production and promotes cell cycle arrest in TCR-activated effector/memory T cells in the presence of sustained TCR signal transduction. *J Immunol* **180**, 1490-1498 (2008).
187. Chen, W. & Wahl, S.M. TGF-beta: the missing link in CD4+CD25+ regulatory T cell-mediated immunosuppression. *Cytokine Growth Factor Rev* **14**, 85-89 (2003).
188. Li, M.O., Wan, Y.Y., Sanjabi, S., Robertson, A.K. & Flavell, R.A. Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol* **24**, 99-146 (2006).
189. Fu, S., *et al.* TGF-beta induces Foxp3 + T-regulatory cells from CD4 + CD25 - precursors. *Am J Transplant* **4**, 1614-1627 (2004).
190. King, C., *et al.* TGF-beta1 alters APC preference, polarizing islet antigen responses toward a Th2 phenotype. *Immunity* **8**, 601-613 (1998).
191. Carel, J.C., Schreiber, R.D., Falqui, L. & Lacy, P.E. Transforming growth factor beta decreases the immunogenicity of rat islet xenografts (rat to mouse) and prevents rejection in association with treatment of the recipient with a monoclonal antibody to interferon gamma. *Proc Natl Acad Sci U S A* **87**, 1591-1595 (1990).
192. Bailey, R. & Griswold, M.D. Clusterin in the male reproductive system: localization and possible function. *Mol Cell Endocrinol* **151**, 17-23 (1999).
193. Dal Secco, V., Riccioli, A., Padula, F., Ziparo, E. & Filippini, A. Mouse Sertoli cells display phenotypical and functional traits of antigen-presenting cells in response to interferon gamma. *Biol Reprod* **78**, 234-242 (2008).
194. Luca, G., *et al.* Improved function of rat islets upon co-microencapsulation with Sertoli's cells in alginate/poly-L-ornithine. *AAPS PharmSciTech* **2**, E15 (2001).
195. Teng, Y., *et al.* Isolation and culture of adult Sertoli cells and their effects on the function of co-cultured allogeneic islets in vitro. *Chin Med J (Engl)* **118**, 1857-1862 (2005).
196. Basta, G., *et al.* Transdifferentiation molecular pathways of neonatal pig pancreatic duct cells into endocrine cell phenotypes. *Transplant Proc* **36**, 2857-2863 (2004).
197. Ferguson, J. & Scothorne, R.J. Extended survival of pancreatic islet allografts in the testis of guinea-pigs. *J Anat* **124**, 1-8 (1977).
198. Selawry, H.P. & Whittington, K. Extended allograft survival of islets grafted into intra-abdominally placed testis. *Diabetes* **33**, 405-406 (1984).
199. Gores, P.F., *et al.* Long-term survival of intratesticular porcine islets in nonimmunosuppressed beagles. *Transplantation* **75**, 613-618 (2003).

200. Dufour, J.M., Rajotte, R.V., Seeberger, K., Kin, T. & Korbutt, G.S. Long-term survival of neonatal porcine Sertoli cells in non-immunosuppressed rats. *Xenotransplantation* **10**, 577-586 (2003).
201. Korbutt, G.S., Elliott, J.F. & Rajotte, R.V. Cotransplantation of allogeneic islets with allogeneic testicular cell aggregates allows long-term graft survival without systemic immunosuppression. *Diabetes* **46**, 317-322 (1997).
202. Dufour, J.M., Rajotte, R.V., Kin, T. & Korbutt, G.S. Immunoprotection of rat islet xenografts by cotransplantation with sertoli cells and a single injection of antilymphocyte serum. *Transplantation* **75**, 1594-1596 (2003).
203. Isaac, J.R., *et al.* Transplantation of neonatal porcine islets and sertoli cells into nonimmunosuppressed nonhuman primates. *Transplant Proc* **37**, 487-488 (2005).
204. Wang, D.*Z., et al.* Xenotransplantation of neonatal porcine islets and Sertoli cells into nonimmunosuppressed streptozotocin-induced diabetic rats. *Transplant Proc* **37**, 470-471 (2005).

CHAPTER 2

SHORT-TERM ADMINISTRATION OF A MONOCLONAL ANTIBODY THERAPY IS EFFECTIVE IN PREVENTING NEONATAL PORCINE ISLET XENOGRAFT REJECTION WHEN ISLETS ARE CO-TRANSPLANTED WITH SERTOLI CELLS

2.1 INTRODUCTION

Since the success of the Edmonton Protocol in 2000 [1], islet transplantation has become a potential method of treating type 1 diabetic patients. However, the need for an unlimited source of islets and a safer method of preventing the rejection of the islet grafts has limited the widespread application of this therapy. To remedy the issue of a shortage of human donor islets, xenotransplantation of neonatal porcine islets (NPI) has been proposed. Xenotransplantation of NPI is an attractive alternative therapy for type 1 diabetic patients because NPI: *i)* are easy to isolate and maintain in culture; *ii)* are capable of reversing hyperglycemia in both small [2] and large animals [3], including non-human primates [4]; and *iii)* have the inherent ability to proliferate and differentiate [5]. Even more, porcine insulin has been used clinically for the treatment of diabetes and is structurally similar to human insulin (differing by one amino acid) [6-8]. To make islet xenotransplantation a clinical reality however, we must first ensure xenograft acceptance by circumventing the potent cellular rejection barrier without chronic administration of harmful immunosuppressive drugs. More specifically, it is important to find short-term therapies that can protect islet grafts and promote immune tolerance.

One potential method of achieving this is to administer short-course treatments of monoclonal antibody (mAb) therapy such as anti-LFA-1, anti-CD154, and anti-CD45RB mAb.

Leukocyte function associated antigen-1 (LFA-1), a β2 integrin, is an attractive target due to its involvement in adhesion, activation, and trafficking of leukocytes [9]. Interaction of LFA-1 with its ligands has been shown to lower the antigen requirements necessary for T cell activation and optimizes T cell/APC contact at the immunological synapse [9,10]. LFA-1 binding to ICAM-1 may also provide co-stimulatory signals to T cells, while blockade of this interaction can lead to the development of tolerance [11]. Thus, mAbs targeting this interaction would affect the ability of T cells to reach their target tissue and be properly activated by antigen presenting cells (APC).

CD154 interaction with CD40 is another effective target for inhibiting T cell activation as it plays a key role in the priming and proliferation of both cytotoxic and helper T cells. This interaction 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 [12]. Blockade of this interaction with anti-CD154 mAb has been proven to be efficacious, providing long-term islet allograft survival in both rodent and non-human primate models [13-16].

Lastly, CD45, a transmembrane protein tyrosine phosphatase expressed on all nucleated hematopoetic cells and their precursors, has been shown to play a critical role in T and B cell receptor signaling as well as in the activation and development of lymphocytes [17]. Blockade of the CD45RB isotype prolongs islet

graft survival in allograft, autoimmune, and concordant xenograft models [18-20]. However, Rayat and Gill have demonstrated that short-term administration of anti-LFA-1, anti-CD45RB, or anti-CD154 mAb is only moderately effective in preventing the rejection of NPI when used as monotherapies [21].

Thus, to provide additional protection to the islet grafts, one potential strategy is to co-transplant islets with Sertoli cells (SC) as an adjunct therapy with the mAbs. Conceptually, this combination would seemingly be effective due to the anti-inflammatory and immunosuppressive properties of testicular SC. These cells have been shown to suppress both T and B cell proliferation and significantly decrease IL-2 production *in vitro* [22]. Though the mechanism of protection has yet to be fully elucidated, it has been suggested that products secreted by SC including FasL [23], TGF-β [24], clusterin [25,26], and serine proteinase inhibitors [27] are responsible for the immunosuppressive and tolerizing properties of SC. Co-transplantation of islets with SC has been shown to have varying protective effects on graft survival in allo- [28], concordant (rat to mouse) and discordant (fish to mouse) xeno- [29,30] , and autoimmune [31] transplant models. In an allograft model, Korbutt et al., showed that 5/5 rats transplanted with allogeneic islets and SC achieved graft survival for >95 days [28]. In contrast, SC alone do not seem sufficient to prevent islet xenograft rejection. In the aforementioned xenograft models, the survival of the concordant xenogeneic islets was greatly enhanced when induction therapy with anti-mouse lymphocyte serum was administered [29], while an alginate microcapsule was used in combination with SC

in the discordant xenograft model [30]. Thus, it appears that SC alone, while protective, are not sufficient to prevent islet xenograft rejection.

In this study, we hypothesized that a monotherapy of mAb in combination with co-transplantation of islets and SC may have a synergistic effect and may result in stable long-term NPI xenograft survival. We report here for the first time that co-transplantation of NPI with SC, in combination with a monotherapy of anti-LFA-1 mAb or anti-CD154 mAb is highly effective in preventing the rejection of NPI in immune-competent mice.

2.2 MATERIALS AND METHODS

2.2.1 Animals

One to 3 day old Duroc and F1 cross neonatal pigs of either sex (1.5-2.5 kg, University of Alberta farm, Alberta, Canada) were used as islet and SC donors respectively. Six to 8 week old male C57BL/6 (B6, H-2^b, Jackson Laboratory, Bar Harbor, ME, USA) mice were used as transplant recipients. Recipient mice were rendered diabetic by a single intraperitoneal injection of streptozotocin (185 mg/kg body weight, Sigma, St Louis, MO, USA) 3-5 days prior to transplantation. Glycemic levels were measured weekly using a One Touch Ultra glucose meter (Lifescan Inc., Milpitas, CA, USA). All diabetic mice had two consecutive non-fasting blood glucose levels >17 mmol/L prior to transplantation. All animals were fed standard laboratory food and cared for according to the guidelines established by the Health Sciences Animal Policy and Welfare Committee at the University of Alberta and the Canadian Council on Animal Care.

2.2.2 Isolation of NPI

Neonatal pigs were anesthetized with halothane and subjected to laparotomy and exsanguination. The pancreas was removed, placed in Hank's balanced salt solution (HBSS) supplemented with 0.25% (w/v) bovine serum albumin (BSA, fraction V; Sigma), chopped into 1-mm fragments, and digested with Type XI collagenase (1 mg/mL; Sigma). Digested tissue was filtered through a 500 μm nylon screen and cultured for 9 days in HAM's F10 medium containing 10 mmol/L D-glucose, 50 μmol/L isobutylmethylxanthine (ICN Biomedicals, Montreal, QC, CA), 0.5% (w/v) 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.

2.2.3 Isolation of Neonatal Porcine SC

One to 3 day old F1 neonatal pigs were anesthetized with halothane. Testicles were removed surgically and placed in HBSS supplemented with 0.25% (w/v) BSA. The testes were chopped into 1-mm fragments after connective tissue lining the testes was removed. Fragments were then digested for 10 min at 37°C with Type XI collagenase (1 mg/mL; Sigma) and washed with HBSS. The tissue was further digested with trypsin (25 μg/mL; Roche, Laval, QC, CA) and DNase (10 μg/mL, Roche) in calcium-free medium supplemented with 1 mmol/L EGTA (ethylene glycol-bis (2-aminoethyl)-N,N,N',N'-tetraacetic acid) and 0.5% (w/v)BSA for 10 minutes at 37°C. Digested tissue was filtered through a 500 μm nylon screen and washed with HBSS. Testicular cells were then co-cultured with

NPI in a 1:1000 ratio (NPI to testicular cells) for 2 days in non-treated Petri dishes (15 cm diameter) containing 35 mL of HAM's F10 medium supplemented with 10 mmol/L D-glucose, 2 mmol/L L-glutamine, 50 μmol/L isobutylmethylxanthine, 0.5% (w/v) BSA, 10 mmol/L nicotinamide, 100 U/mL penicillin, and 100 μg/mL streptomycin for two days at 37°C, 5% CO_2, and 95% air.

2.2.4 Transplantation of NPI and Neonatal Porcine SC

After two days of culture, NPI alone or NPI and SC were collected and transplanted under the left kidney capsule of streptozotocin-induced diabetic B6 mice. As previously described by Korbutt et al., aliquots of the NPI alone or NPI with SC were counted to determine the number of islet equivalents in each preparation (one islet equivalent is equal to 150μm). Aliquots of 2000 NPI equivalents were prepared and aspirated into polyethylene tubing (PE-50). Islets were pelleted via centrifugation and placed within the subcapsular pocket of the kidney using a micromanipulator syringe. The kidney capsule was then cauterized. Graft function in recipient mice was indicated by blood glucose values <10 mmol/L, while graft rejection was determined as the first of three consecutive readings of blood glucose values >10 mmol/L. Graft rejection was further determined by histological analysis of the graft. To ensure that the normoglycemic state was graft-dependent, recipients with long-term (>100 days) graft survival underwent a survival nephrectomy of the graft-bearing kidney to see if recipients would revert to hyperglycemic state.

2.2.5 Anti-rejection Therapies

Transplanted streptozotocin-induced diabetic B6 mice were randomly selected to receive either no mAb treatment or one of the following mAb therapies intraperitoneally: 1) anti–LFA-1 mAb (KBA; rat IgG2a; kindly provided by Dr. Ron G. Gill, University of Alberta) at 200 μg on days 0, 1, 7, 14 post-transplant; 2) anti-CD154 mAb (MR-1; hamster IgG1; BioExpress, West Lebanon, NH, USA) at 250 μg on days -1, 1, 4, 7, 11, 14, 18, 21, 25, 28 post-transplant; or 3) anti-CD45RB mAb (HB220; Rat IgG2a; BioExpress) at 300 μg on day -1, and 100 μg on days 0, 1, 2, 3, 4, and 5 post-transplant.

2.2.6 Immunohistological Analysis of Graft Sections

Graft-bearing kidneys were harvested following rejection or at the end of the study (>100 days post-transplant), fixed with 10% (w/v) buffered formalin solution, and embedded in paraffin. Graft sections (5 μm thick) were stained to determine the presence of insulin producing cells, SC, and infiltrating immune cells. For insulin, sections were quenched in 10% (v/v) hydrogen peroxide, blocked with 20% (v/v) normal goat serum (Cedarlane, Burlington, ON, CA), and then incubated for 30 minutes with guinea pig anti-insulin antibody (1:1000 dilution, Dako Laboratories, Mississauga, ON, CA). This was followed by a 30 minute incubation with biotinylated goat anti-guinea pig IgG secondary antibody (1:200 dilution, Vector Laboratories, Burlingam, CA, USA). The brown color was produced with an avidin-biotin complex/horseradish peroxidase (ABC/HP, Vector Laboratories) and 3, 3-diaminobenzidinetetrahydrochloride (DAB,

BioGenex, San Ramon, CA, USA). Graft sections were then counterstained with Harris' hematoxylin and eosin.

For the identification of SC, graft sections were stained for vimentin or Müllerian inhibiting substance (MIS). Antigen retrieval was performed by microwaving graft sections in 0.01 mol/L sodium citrate buffer. Sections were quenched in 10% (v/v) hydrogen peroxide, blocked with 20% (v/v) normal goat serum (Cedarlane) for vimentin staining or 20% (v/v) normal rabbit serum (Cedarlane) for MIS staining, and incubated with either mouse anti-vimentin (1:100 dilution, Dako) or goat anti-MIS (1:25 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA). They were then incubated for 20 minutes with either biotinylated goat anti-mouse secondary antibody (1:200 dilution, Cederlane) or biotinylated horse anti-goat secondary antibody (1:200 dilution, Vector Laboratories), respectively. The brown color was produced with avidin-biotin complex/horseradish peroxidase and 3, 3-diaminobenzidinetetrahydrochloride. Slides were then counterstained with Harris' hematoxylin.

Subpopulations of immune cells were characterized from cryosections of the islet grafts immersed in OCT compound and snap frozen at -80^{o}C. Sections were air dried for 4 minutes at room temperature and then fixed in acetone at 4^{o}C for 3 minutes. Sections that were stained for foxp3 were then permeabilized using 0.1% (v/v) Triton-X100 solution (in PBS) for 10 minutes. This step was not done for other immune cell staining. All sections were then blocked with a 2% (v/v) FBS/PBS solution. An avidin/biotin blocking kit was subsequently used to block endogenous biotin or biotin-binding proteins (Vector Laboratories). Sections were

then incubated for 45 minutes with rat anti-mouse CD4 (1:100; BD Pharmingen, Mississauga, ON, CA), rat anti-mouse CD8 (1:100; BD Pharmingen), rat anti-mouse CD19 (1:200; BD Pharmingen), or rat anti-mouse CD11b (1:500; BD Pharmingen) antibody, or were incubated for 1 hour with rat anti-mouse/rat foxp3 (eBioscience, San Diego, CA, USA) antibody. After washing with 2% (v/v) FBS/PBS solution, sections were incubated for 30 minutes with biotinylated rabbit anti-rat IgG secondary antibody (1:200, Vector Laboratories). The brown color was produced with an avidin-biotin complex/horseradish peroxidase and 3, 3-diaminobenzidinetetrahydrochloride. Sections were counterstained with Harris' hemotoxylin. Stained slides were visualized with an Axioscope II microscope equipped with an Axiocam MRC camera, and were analyzed using Axiovision 4.6 software (Carl Zeiss, Göttingen, Germany).

2.2.7 Detection of Mouse Anti-porcine Antibody Levels in Mouse Serum

To determine the effect of various mAb therapies in mice co-transplanted with NPI and neonatal porcine SC on the production of mouse anti-porcine antibodies, mouse anti-porcine IgG levels in the blood serum of each transplant recipient was determined using flow cytometry. Peripheral blood samples from B6 mice were collected and their sera were isolated. Porcine spleen cells (1×10^6) obtained from the same pig islet donors were incubated with diluted mouse serum (1:128 dilution) for 1 hour at 37°C, 5% CO_2, and 95% air. Spleen cells were then washed with PBS and incubated with goat anti-mouse IgG FITC labeled antibody (1:200 dilution, Southern Biotechnology Associates, Inc., Birmingham, AL, USA)

for 1 hour at 4°C. Ten thousand events were taken from each sample, gated on viable spleen cells. The percentage of cells bound to antibody was detected from single parameter fluorescence histograms on a BD FACs Calibur machine (BD Biosciences, Mississauga, ON, CA). An M1 gate was used to determine the percentage of cells fluorescing above control (background) levels. Thus, the overall production of anti-porcine antibody was assessed by analyzing the proportion/percentage of cells that were bound by anti-porcine antibody at levels above background. Controls for this experiment include pig spleen cells incubated without serum or secondary antibody (unstained) and pig spleen cells incubated with secondary antibody without mouse serum.

2.2.8 Statistical Analysis

Statistical differences in graft survival among the various treatment groups was assessed using the Kaplan-Meier Log rank test while statistical differences for serum anti-porcine antibody levels were determined using the Mann-Whitney U Test. A p-value < 0.05 was considered to be statistically significant.

2.3 RESULTS

2.3.1 Co-transplantation of NPI with Neonatal Porcine SC is Not Sufficient to Prevent NPI Xenograft Rejection

Neonatal porcine SC were co-transplanted with NPI to determine the efficacy of SC in preventing islet xenograft rejection in our model. We found that none of the B6 recipients receiving NPI co-transplanted with SC in the absence of

mAb treatment (n=8) achieved normoglycemia (Table 2.1 and Figure 2.1) after transplantation. One mouse died on day 72 and another on day 85 post-transplant due to hyperglycemia. The same result was seen in mice transplanted with NPI alone (n=5) suggesting that SC alone cannot prevent the rejection of NPI in immune-competent B6 mice. This was supported by histological examination of the grafts which show a complete absence of islet graft tissue (Figure 2.2 and Figure 2.3) in both of these groups.

Table 2.1: Graft survival in B6 mice transplanted with NPI or NPI and SC treated with mAb

Treatment	N	Graft Survival (Days)	Number of Recipients Achieving Normoglycemia
NPI Alone	5	0 (x 5)	0/5
Anti-LFA-1 mAb	7	0 (x 3), 99, >100 (x 3)	4/7†
Anti-CD154 mAb	7	0 (x 5), >100 (x 2)	2/7
Anti-CD45RB mAb	7	0 (x 6), >100 (x 1)	1/7
NPI + SC Alone	8	0 (x 8)	0/8
Anti-LFA-1 mAb + SC	7	>100 (x 7)	7/7‡
Anti-CD154 mAb + SC	8	0, >100 (x 7)	7/8*‡
Anti-CD45RB mAb + SC	9	0 (x 3), 81, 82, >100 (x 4)	6/9‡

Streptozotocin-induced diabetic (blood glucose level >17.0 mmol/L) B6 mice were transplanted with 2000 NPI alone or 2000 NPI co-cultured with $2x10^6$ neonatal porcine testicular cells under the kidney capsule. Recipient mice were left untreated or were treated with a short-course of anti-LFA-1 mAb (KBA; rat IgG2a; 200 µg administered on days 0, 1, 7, 14 post-transplant), anti-CD154 mAb (MR-1; hamster IgG1; 250 µg on days -1, 1, 4, 7, 11, 14, 18, 21, 25, and 28 post-

transplant), or anti-CD45RB mAb (HB220; rat IgG2a; 300 µg/mouse on day -1 and 100 µg/mouse on days 0, 1, 2, 3, 4, and 5 post-transplant). Blood glucose levels of these recipient mice were monitored weekly for reversal of diabetes. Graft survival of 0 indicates the inability of the transplanted islets to reverse hyperglycemia in the recipient mouse.
* $p < 0.05$ NPI + SC with mAb therapy compared to NPI alone with the same mAb treatment.
† $p < 0.05$ NPI with mAb therapy compared to NPI alone.
‡ $p < 0.05$ NPI + SC with mAb therapy compared to NPI + SC alone.

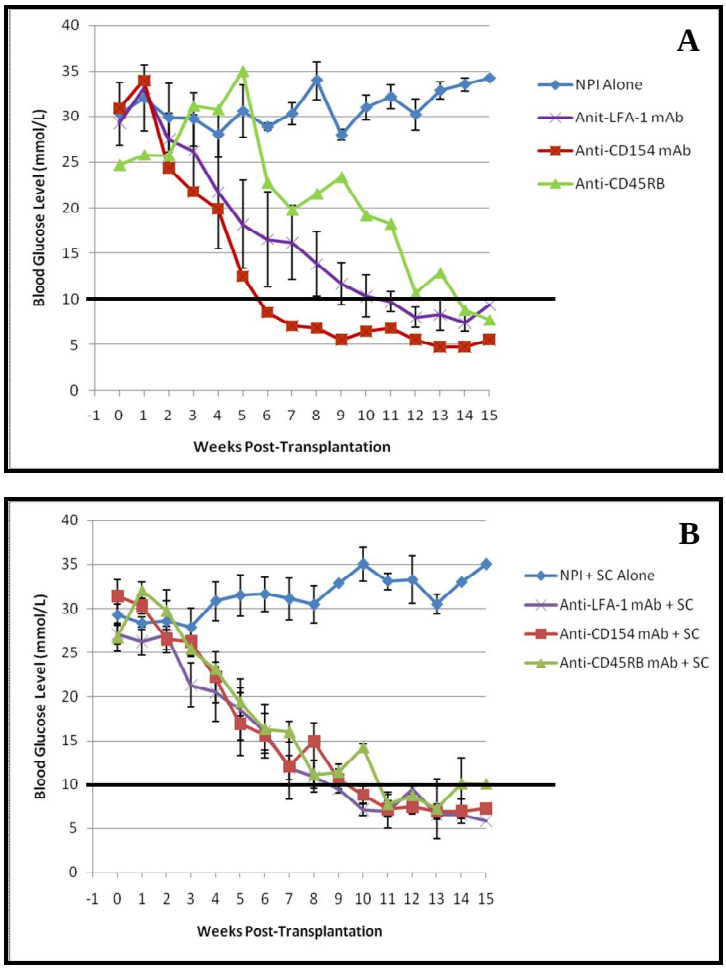

Figure 2.1: Average blood glucose levels of B6 mice transplanted with NPI alone or NPI with neonatal porcine SC treated with a mAb monotherapy. Streptozotocin-induced diabetic B6 mice (blood glucose level > 17.0 mmol/L) received an islet graft alone or co-transplanted with SC under the kidney capsule. Blood glucose levels were monitored weekly thereafter. Return to euglycemia (blood glucose level < 10 mmol/L) did not occur in mice that received NPI alone without mAb therapy, but did occur in 4 mice treated with anti-LFA-1 mAb, in 2

mice treated with anti-CD154 mAb, and in 1 mouse treated with anti-CD45RB mAB between 5 and 14 weeks post-transplantation (A). Recipient mice receiving NPI and SC without mAb were also unable to achieve normoglycemia, however 7 mice treated with anti-LFA-1 mAb, 7 mice treated with anti-CD154 mAb, and 6 mice treated with anti-CD45RB mAb that received NPI and SC achieved normoglycemia between 4 to 15 weeks post-transplantation.

2.3.2 Co-transplantation of NPI with SC Combined with mAb Therapy Prolongs NPI Xenograft Survival

To assess if co-transplantation of NPI and SC combined with a mAb therapy is effective in preventing NPI xenograft rejection, we transplanted mice with either NPI alone or NPI with SC. These mice then received a short-course treatment of anti-LFA-1, anti-CD154, or anti-CD45RB mAb. In mice transplanted with NPI alone, 3/7 (43%) mice treated with anti-LFA-1 mAb, 2/7 (29%) mice treated with anti-CD154 mAb, and 1/7 (14%) mice treated with anti-CD45RB mAb achieved long-term graft survival (>100 days).

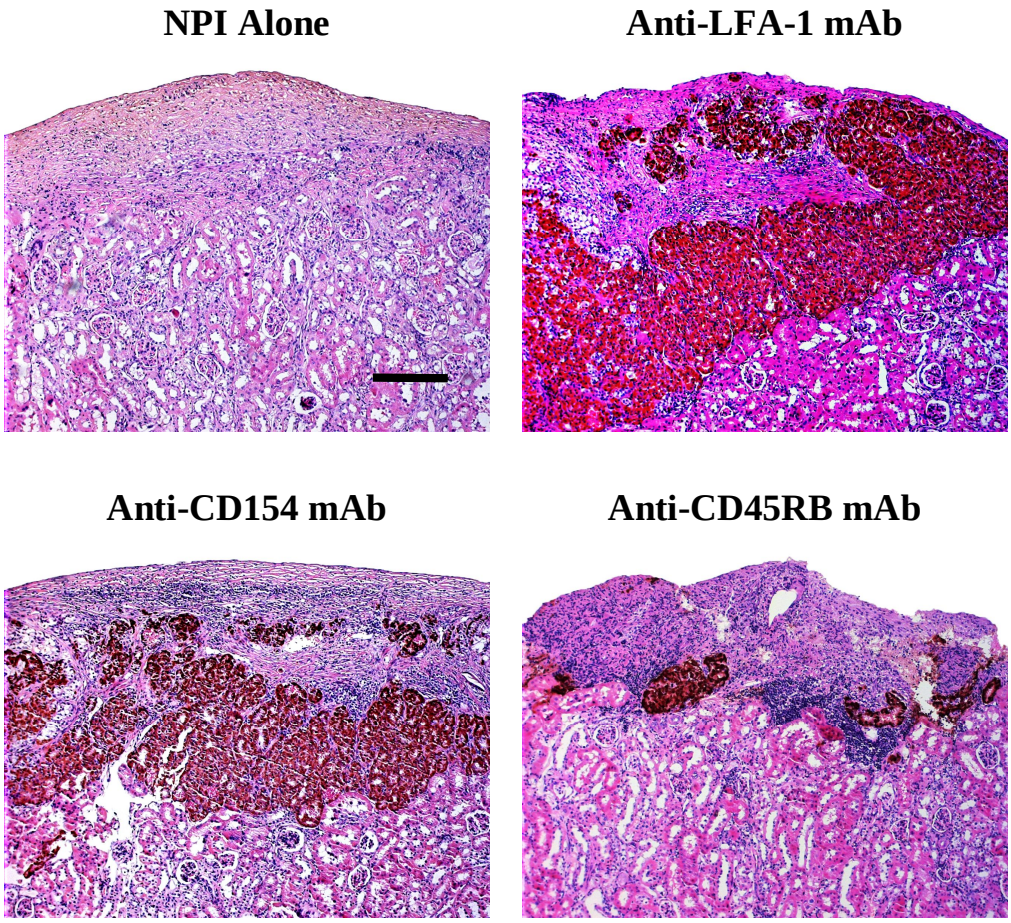

Figure 2.2: Immunohistochemical analysis of grafts recovered from mAb treated mice transplanted with NPI alone. Graft-bearing kidneys were harvested from mice at >100 days post-transplantation. Kidneys were then embedded in paraffin and sectioned. Histological examination of the xenografts at >100 days post-transplant shows the presence of insulin positive cells in the grafts of treated recipients achieving long-term graft survival, whereas a complete absence of insulin producing tissue is evident in mice transplanted with NPI alone without mAb therapy. Scale bar represents 200 μm.

These proportions however, were considerably improved when NPI were co-transplanted with SC, as 7/7 (100%) mice treated with anti-LFA-1 mAb, 7/8 (88%) mice treated with anti-CD154 mAb, and 4/9 (44%) mice treated with anti-CD45RB mAb achieved long-term graft survival. Two of these mice that were treated with anti-CD45RB mAb achieved normoglycemia, but rejected their grafts

on days 81 and 82 post-transplant. To ensure that the normoglycemic state was graft-dependent in mice that achieved long-term graft survival, nephrectomies of the graft-bearing kidneys were performed at >100 days post-transplant. All protected mice reverted back to hyperglycemia post-nephrectomy.

Histological examination of the islet grafts from protected mice that received NPI alone with mAb therapy shows the presence of insulin positive cells, whereas both insulin positive (islet β cells) and vimentin and MIS positive cells (SC) were clearly present in treated mice receiving NPI and SC (Figure 2.3). However, while normal islet morphology was clearly seen in protected mice receiving NPI and mAb alone, islets in mice receiving NPI and SC are more fragmented and scattered throughout the graft tissue. Consistent with their morphology in the native testis, vimentin and MIS staining showed that SC form tubular-like structures within the graft. Interestingly, mononuclear cell infiltrates were also observed in the grafts of all recipients, regardless of the treatment they received.

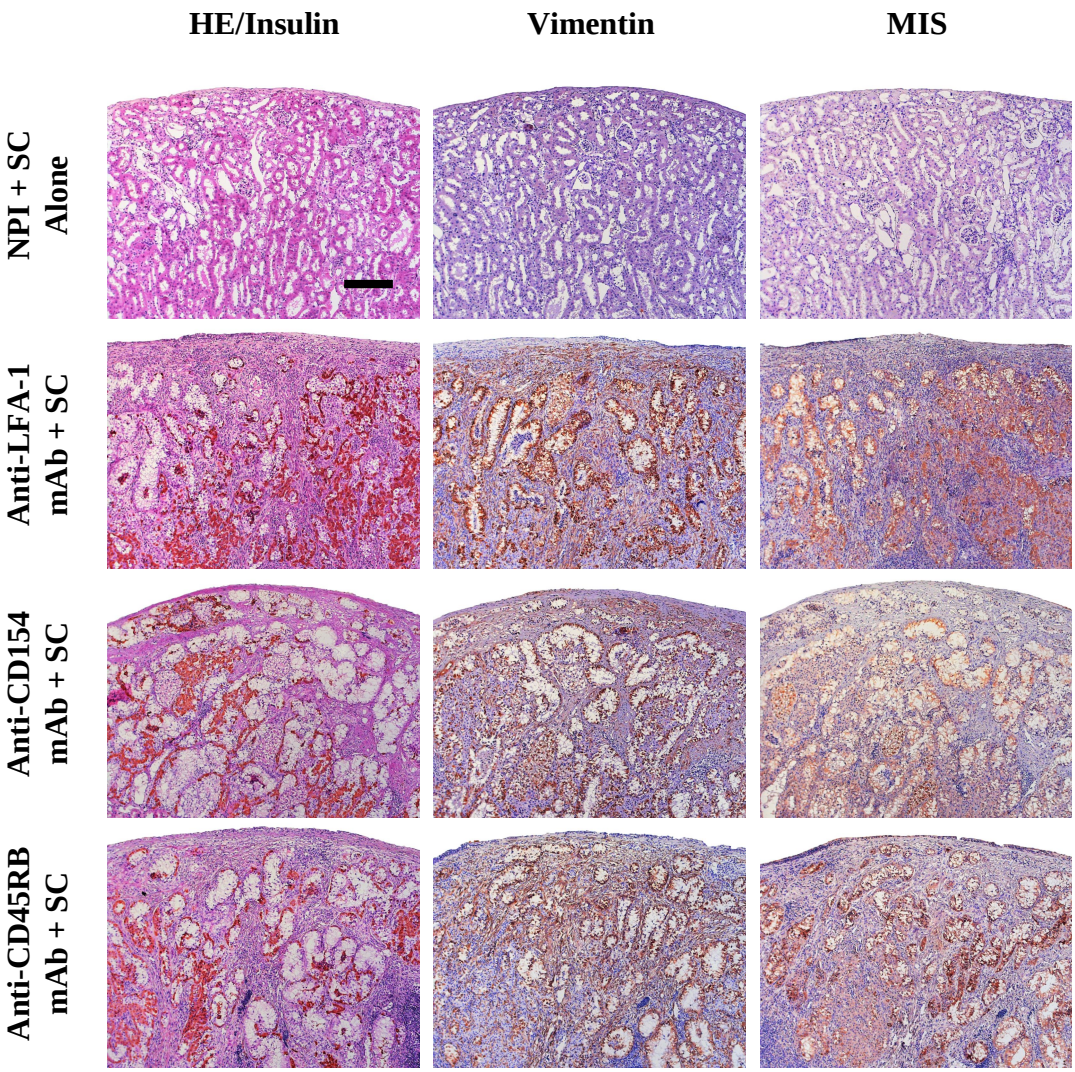

Figure 2.3: Gross morphology of NPI co-transplanted with neonatal porcine SC into B6 mice assessed by immunohistochemistry staining. Graft-bearing mice were euthanized at >100 days post-transplant and porcine islet and SC xenografts were stained for insulin, vimentin, and MIS. Histological examination of the xenografts at >100 days post-transplantation shows an absence of transplanted tissue in untreated recipient mice. In contrast, both insulin positive cells and SC are seen in protected mice that received a monotherapy of mAb. Scale bar represents 200 µm.

2.3.3 Characterization of Immune Cell Infiltrate within Grafts of Recipient Mice

In order to characterize the immune cell infiltrates observed within grafts of recipient mice, we stained for the presence of $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, and $CD11b^+$ macrophages using immunohistochemistry (Figure 2.4). Regardless of the treatment, we found that the phenotype of infiltrating immune cells were consistent between all groups. Immunohistochemical analysis revealed a dominant presence of $CD4^+$ T cells distributed throughout the remaining graft tissue of recipient mice. In contrast, relatively few $CD8^+$ T cells were present. A significant presence of $CD19^+$ B cells was also seen in the graft tissue, but remained primarily within areas of dense mononuclear cell infiltrate rather than diffusely spread as seen with $CD4^+$ T cells. Lastly, $CD11b^+$ macrophages were also low in numbers within the graft site.

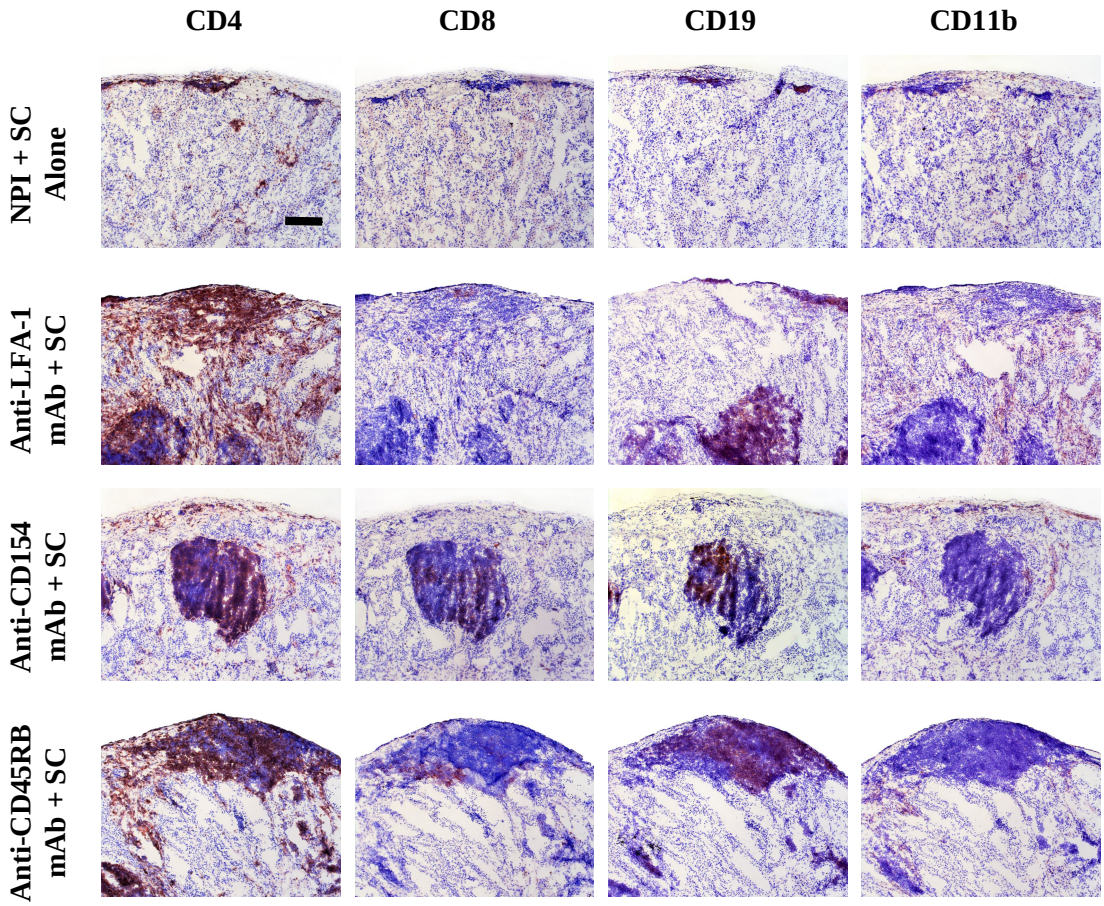

Figure 2.4: Characterization of the immune cell infiltrate within islet grafts of mice transplanted with NPI and SC. At the time of rejection, or at >100 days post-transplantation, recipient mice underwent nephrectomies to recover the graft-bearing kidneys. Grafts were stained for the presence of $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells, and $CD11b^+$ macrophages. Regardless of treatment, there was a prominence of $CD4^+$ T cells and $CD19^+$ B cells, with a relatively low numbers of $CD8^+$ T cells and $CD11b^+$ macrophages. Scale bar represents 200 μm.

2.3.4 Presence of Foxp3$^+$ Cells within Graft Tissue

As the immune cell infiltrate mentioned above was consistent between all groups, we had little idea about the mechanism of protection in the mice that had prolonged graft survival. Thus, we wanted to determine whether regulatory T cells were present within the grafts of protected mice. To do this, we stained for

the presence of foxp3^{+} cells locally within the graft site. Here we found that in comparison to mice that had rejected their graft, relatively large numbers of foxp3^{+} cells were apparent within the grafts of mice that had prolonged graft survival (Figure 2.5). This suggests that the foxp3^{+} cells may be an important element of the protection induced by the combination of SC and mAb therapy.

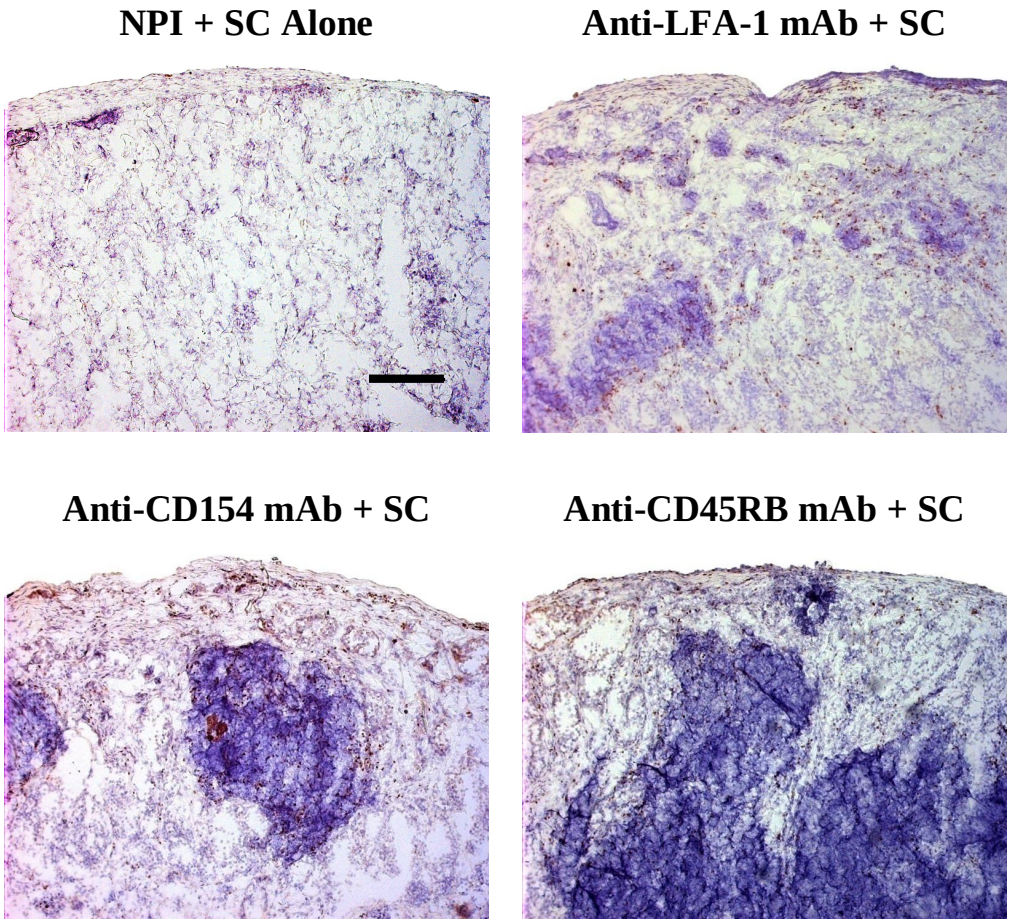

Figure 2.5: Representative foxp3 immunohistochemical staining of grafts from mice that received islets and SC. Grafts harvested from mice with NPI and SC xenografts without mAb therapy at >100 days post-transplantation showed little to no evidence of foxp3^{+} cellular infiltrate within the graft site. However, foxp3^{+} cells are clearly evident in grafts from treated mice achieving prolonged graft survival, suggesting that T regulatory cells may be involved in the protection seen in treated mice. Scale bar represents 200 μm.

2.3.5 Levels of Anti-porcine Antibody in Recipient Mice Treated with Anti-LFA-1, Anti-CD154, or Anti-CD45RB mAb

To assess the anti-donor reactivity towards NPI co-transplanted with SC under the various mAb treatments, the relative production of mouse anti-porcine IgG antibody was examined using flow cytometry. Here, the levels of anti-pig IgG antibodies was significantly ($p<0.05$) decreased with anti-CD154 mAb therapy. In contrast, there was no overall decrease in anti-porcine antibody levels in mice that received anti-LFA-1 mAb, as shown in Figure 2.6. Antibody production in mice treated with anti-CD45RB mAb was highly variable but was related to graft rejection. This was consistent with the antibody production in protected mice receiving NPI alone and mAb therapy, where a low anti-pig antibody response was observed in mice that were normoglycemic prior to euthenization, whereas high antibody production was evident in mice that rejected their grafts. The exception to this was in mice treated with anti-CD154 mAb where low levels of antibody were detected regardless of rejection.

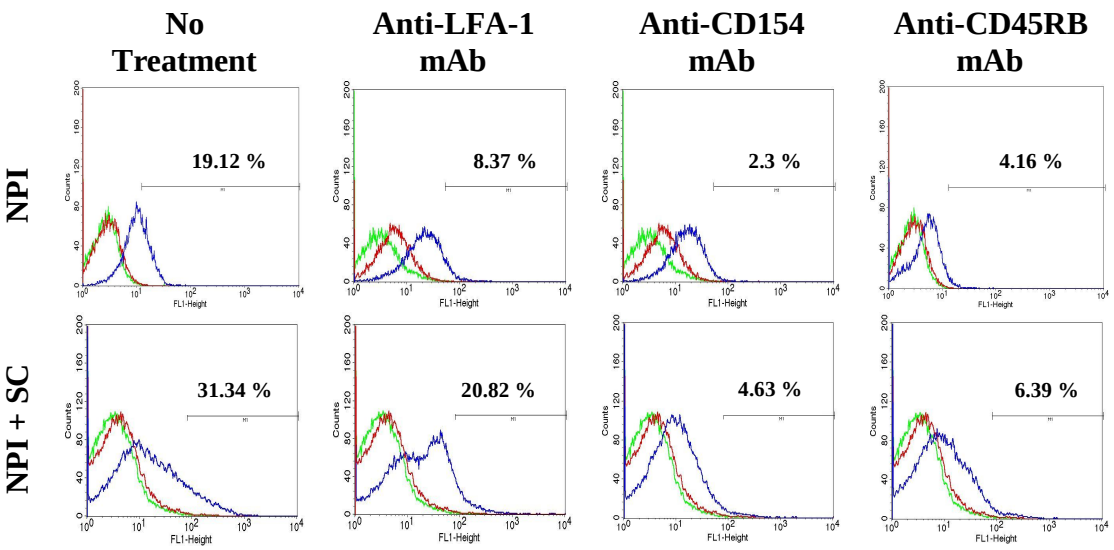

Figure 2.6: Representative analysis of anti-porcine antibody production in mAb treated B6 mice that were transplanted with NPI or NPI and SC. Porcine spleen cells (1 x 10^6) obtained from the same pig islet donors were incubated with mouse serum (1:128 dilution) obtained from each mouse transplanted with NPI or NPI and SC that received no treatment, anti-LFA-1 mAb, anti-CD154 mAb, or anti-CD45RB mAb treatment. Levels of bound anti-porcine IgG were measured and analyzed via flow cytometry. Controls included unstained cells (represented by red line) and cells incubated with secondary antibody alone without mouse serum (represented by green line). The M1 gate was used to distinguish cells bound by anti-porcine antibodies from unstained and/or background staining. Thus, the percentage of cells within the M1 gate represents the percentage of cells bound by mouse anti-porcine antibody.

2.4 DISCUSSION

Rayat and Gill have previously shown that when NPI are transplanted into B6 mice, only 6/15 mice treated with anti-LFA-1 mAb, 2/7 mice treated with anti-CD154 mAb, and 2/8 mice treated with anti-CD45RB mAb, achieved long-term graft survival [21]. Similar results were found in our study as 4/7 mice treated with anti-LFA-1 mAb, 2/7 mice treated with anti-CD154 mAb, and 1/7 mice treated with anti-CD45RB mAb, achieved long-term graft survival. For the first time

however, we have shown that long-term islet xenograft survival can be achieved in a discordant islet xenograft model using a single mAb therapy when NPI are co-transplanted with SC.

While SC have been shown to be protective to islet grafts in allogeneic [28], autoimmune [31], and xenogeneic animal models (rat to mouse and fish to mouse) of transplantation [29,30], our study demonstrated that alone, co-transplantation of NPI with SC is insufficient in preventing the rejection of the NPI xenografts. This was confirmed by a complete absence of graft tissue in mice that received NPI and SC at >100 days post-transplantation. This result is consistent with previous studies using NPI and neonatal porcine SC [32,33], and other islet xenograft models, where an additional strategy such as alginate microencapsulation [30] or induction therapy with anti-lymphocyte serum [29] was required to prolong the survival of the islets. It has been suggested that these additional strategies are necessary to provide sufficient time for SC to engraft and become functional [29].

Indeed, in our study we found a marked improvement in the proportion of mice that achieved normoglycemia and long-term graft survival when a monotherapy of anti-LFA-1, anti-CD154 or anti-CD45RB mAb was administered to recipients of NPI and SC. Most notably 100% of the recipients injected with anti-LFA-1 mAb and 88% of recipients injected with anti-CD154 mAb achieved and maintained normoglycemia long-term. This is a considerable improvement compared to mAb monotherapy alone; indicating that SC are able to provide additional protection to the NPI xenografts. Three important molecules secreted

by SC which are hypothesized to provide protection to islet grafts are TGF-β, FasL, and clusterin.

TGF-β has emerged as a key regulator of immune responses and is well known for its immunomodulatory and anti-inflammatory functions, inhibiting both T cell proliferation [34] and IL-2 production [35]. TGF-β has also been implicated in playing a major role in T regulatory cell mediated suppression, immune tolerance, and in the induction of foxp3^{+} T regulatory cells from CD4^{+}CD25^{-} precursors [36-38]. As a result, TGF-β has been proposed as a critical feature in SC mediated protection of co-transplanted islets [24]. This potentiation of the induction of regulatory cells and anti-inflammatory effects of TGF-β combined with the inhibition of immune cell activation by mAb therapies provides a logical basis for the synergistic effect seen with this combination of anti-rejection strategies.

Even more, the elimination of activated lymphocytes with FasL may prevent potentially reactive cells from initiating immune rejection. The role of FasL has been proposed as a major mechanism through which immune privilege is maintained in the testis [23,39], though its role in islet graft protection remains controversial [25]. Engagement of FasL with its receptor Fas has been shown to initiate apoptosis of Fas expressing cells [40]. Thus, due to the up-regulation of Fas on activated lymphocytes, SC secretion of FasL may protect islet tissue by destroying potentially destructive activated T cells.

Clusterin has also demonstrated potent anti-rejection properties, and is one of the most abundant molecules secreted by SC. In an *in vitro* study by Dufour et al., it was proposed that clusterin effectively blocked membrane attack complex

(MAC) formation and thus, prevented complement mediated destruction and humoral mediated rejection [26]. This combined with the inhibition of antibody production by mAb therapies (specifically anti-CD154 mAb and anti-CD45RB mAb when combined with SC) is another mechanism through which mAb therapies and SC may complement one another.

Histological examination of the grafts from mice with long-term normoglycemia clearly shows that both insulin positive cells as well as SC are present within the graft. As previously described, SC seem to form tubule-like structures within the graft [41]. It is interesting to note that a large portion of the insulin positive cells seen in the grafts are fragmented around these tubule-like structures. While the formation of these structures is likely important in the proper functioning of the SC [42], it is unclear whether this fragmentation has a negative impact on the long-term survival and function of the islets. Thus, studies which examine the effect of these tubule-like structures on the viability and function of the islets will need to be performed.

Moreover, it is interesting to note that dense mononuclear cell infiltrates are apparent in all treatment groups, including grafts from protected mice. These cellular infiltrates may actively be rejecting the islet graft, may be protective, or may in some way be regulated or rendered ineffective. Analysis of the phenotypes of these cells demonstrated no differences in the proportion of $CD4^+$, $CD8^+$, $CD19^+$, and $CD11b^+$ cells, regardless of treatment or status of rejection. Here, immunohistochemical analysis revealed a heavy infiltrate of $CD4^+$ T cells and $CD19^+$ B cells, with fewer $CD8^+$ T cells and $CD11b^+$ macrophages. While the

consistency of the infiltrate mentioned above reveals little about the possible mechanism of protection seen in mice achieving long-term graft survival, we also sought to determine whether there was a local presence of foxp3$^+$ T regulatory cells within the grafts. In comparison to rejected mice, we found that recipients that had prolonged graft survival contained foxp3$^+$ cells, indicating that T regulatory cells may provide some level of protection within the graft site. This is consistent with other recent studies that have shown an enhanced proportion of suppressive CD4$^+$CD25$^+$foxp3$^+$ T regulatory cells when SC are co-cultured with lymphocytes [43], or when islets were transplanted into the testicular environment of diabetic mice [44]. These studies suggest that regulatory T cells may be involved in the protection induced by SC.

Our data also shows that mAb therapies were effective in reducing anti-donor reactivity as demonstrated by the inhibition of antibody production against porcine tissue. In general however, this reduction was dependent on the status of rejection as recipients that recently rejected their NPI or NPI and SC grafts had considerably higher levels of antibody production compared to mice that had not rejected their graft. There were two major exceptions to this. The first is that low antibody production was seen regardless of rejection in mice that received anti-CD154 mAb treatment. This is most likely attributable to the critical role of CD40 ligation with CD154 in the maturation of B cells and in class switching, thus this observation could be expected [45-47]. The second exception was in mice receiving NPI and SC that were treated with anti-LFA-1 mAb. These mice had considerable variability in anti-porcine antibody production which seemed irrespective of graft

rejection, as all of these recipients achieved graft survival for the entire follow-up period. This was in contrast to anti-LFA-1 mAb treated mice that received NPI alone, where mice that were normoglycemic at the endpoint of the study all showed low levels of anti-porcine antibody levels. This suggests that the additional antigens present due to the transplantation of SC may have enhanced reactivity towards the graft and that anti-LFA-1mAb was less able to suppress this humoral response compared to the other therapies. Even then, mice that were co-transplanted with NPI and SC that were treated with anti-LFA-1 mAb all achieved and maintained graft survival until the endpoint of the study. Thus, other mechanisms such as inhibition of complement mediated destruction may have provided additional protection to the graft to overcome this enhanced reactivity. Further studies will need to be done to determine whether these mice were actively rejecting their graft or if graft protection is stable despite the increased antibody production.

Overall, our results indicate that co-transplantation of NPI with SC in combination with a short-course treatment of mAb monotherapy can be effective in preventing the rejection of NPI xenografts. While the objective of this experiment was to determine the efficacy of this combination of strategies, we may have also found a way to avoid using anti-CD154 mAb, which, due to potential thromboembolic side effects will likely not be clinically available. On the other hand, the humanized form of anti-LFA-1 mAb (Efalizumab) is currently being used to treat human patients with psoriasis vulgaris. Thus, incorporation of

anti-LFA-1 mAb as a part of the anti-rejection regime for islet xenotransplantation is very attractive.

2.5 REFERENCES

1. Shapiro, A.M., *et al.* Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* **343**, 230-238 (2000).
2. Korbutt, G.S., *et al.* Large scale isolation, growth, and function of porcine neonatal islet cells. *J Clin Invest* **97**, 2119-2129 (1996).
3. Kin, T., Korbutt, G.S., Kobayashi, T., Dufour, J.M. & Rajotte, R.V. Reversal of diabetes in pancreatectomized pigs after transplantation of neonatal porcine islets. *Diabetes* **54**, 1032-1039 (2005).
4. Cardona, K., *et al.* Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat Med* **12**, 304-306 (2006).
5. Rayat, G.R., Rajotte, R.V. & Korbutt, G.S. Potential application of neonatal porcine islets as treatment for type 1 diabetes: a review. *Ann N Y Acad Sci* **875**, 175-188 (1999).
6. Socci, C., *et al.* Selection of donors significantly improves pig islet isolation yield. *Horm Metab Res Suppl* **25**, 32-34 (1990).
7. Smith, L.F. Species variation in the amino acid sequence of insulin. *Am J Med* **40**, 662-666 (1966).
8. Ruttenberg, M.A. Human insulin: facile synthesis by modification of porcine insulin. *Science* **177**, 623-626 (1972).
9. Nicolls, M.R. & Gill, R.G. LFA-1 (CD11a) as a therapeutic target. *Am J Transplant* **6**, 27-36 (2006).
10. Bachmann, M.F., *et al.* Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity* **7**, 549-557 (1997).
11. Nicolls, M.R., Coulombe, M., Yang, H., Bolwerk, A. & Gill, R.G. Anti-LFA-1 therapy induces long-term islet allograft acceptance in the absence of IFN-gamma or IL-4. *J Immunol* **164**, 3627-3634 (2000).
12. Xu, Y. & Song, G. The role of CD40-CD154 interaction in cell immunoregulation. *J Biomed Sci* **11**, 426-438 (2004).
13. Parker, D.C., *et al.* Survival of mouse pancreatic islet allografts in recipients treated with allogeneic small lymphocytes and antibody to CD40 ligand. *Proc Natl Acad Sci U S A* **92**, 9560-9564 (1995).
14. Rossini, A.A., *et al.* Induction of immunological tolerance to islet allografts. *Cell Transplant* **5**, 49-52 (1996).
15. Kenyon, N.S., *et al.* Long-term survival and function of intrahepatic islet allografts in rhesus monkeys treated with humanized anti-CD154. *Proc Natl Acad Sci U S A* **96**, 8132-8137 (1999).
16. Kenyon, N.S., *et al.* Long-term survival and function of intrahepatic islet allografts in baboons treated with humanized anti-CD154. *Diabetes* **48**, 1473-1481 (1999).
17. Penninger, J.M., Irie-Sasaki, J., Sasaki, T. & Oliveira-dos-Santos, A.J. CD45: new jobs for an old acquaintance. *Nat Immunol* **2**, 389-396 (2001).

18. Auersvald, L.A., *et al.* Indefinite islet allograft survival in mice after a short course of treatment with anti-CD45 monoclonal antibodies. *Transplantation* **63**, 1355-1358 (1997).
19. Molano, R.D., *et al.* Prolonged islet graft survival in NOD mice by blockade of the CD40-CD154 pathway of T-cell costimulation. *Diabetes* **50**, 270-276 (2001).
20. Visser, L., *et al.* Prolonged survival of rat islet xenografts in mice after CD45RB monotherapy. *Transplantation* **77**, 386-391 (2004).
21. Rayat, G.R. & Gill, R.G. Indefinite survival of neonatal porcine islet xenografts by simultaneous targeting of LFA-1 and CD154 or CD45RB. *Diabetes* **54**, 443-451 (2005).
22. De Cesaris, P., *et al.* Immunosuppressive molecules produced by Sertoli cells cultured in vitro: biological effects on lymphocytes. *Biochem Biophys Res Commun* **186**, 1639-1646 (1992).
23. Bellgrau, D., *et al.* A role for CD95 ligand in preventing graft rejection. *Nature* **377**, 630-632 (1995).
24. Suarez-Pinzon, W., *et al.* Testicular sertoli cells protect islet beta-cells from autoimmune destruction in NOD mice by a transforming growth factor-beta1-dependent mechanism. *Diabetes* **49**, 1810-1818 (2000).
25. Dufour, J.M., Rajotte, R.V., Korbutt, G.S. & Emerich, D.F. Harnessing the immunomodulatory properties of Sertoli cells to enable xenotransplantation in type I diabetes. *Immunol Invest* **32**, 275-297 (2003).
26. Dufour, J.M., Hamilton, M., Rajotte, R.V. & Korbutt, G.S. Neonatal porcine Sertoli cells inhibit human natural antibody-mediated lysis. *Biol Reprod* **72**, 1224-1231 (2005).
27. Sipione, S., *et al.* Identification of a novel human granzyme B inhibitor secreted by cultured sertoli cells. *J Immunol* **177**, 5051-5058 (2006).
28. Korbutt, G.S., Elliott, J.F. & Rajotte, R.V. Cotransplantation of allogeneic islets with allogeneic testicular cell aggregates allows long-term graft survival without systemic immunosuppression. *Diabetes* **46**, 317-322 (1997).
29. Dufour, J.M., Rajotte, R.V., Kin, T. & Korbutt, G.S. Immunoprotection of rat islet xenografts by cotransplantation with sertoli cells and a single injection of antilymphocyte serum. *Transplantation* **75**, 1594-1596 (2003).
30. Yang, H. & Wright, J.R., Jr. Co-encapsulation of Sertoli enriched testicular cell fractions further prolongs fish-to-mouse islet xenograft survival. *Transplantation* **67**, 815-820 (1999).
31. Korbutt, G.S., Suarez-Pinzon, W.L., Power, R.F., Rajotte, R.V. & Rabinovitch, A. Testicular Sertoli cells exert both protective and destructive effects on syngeneic islet grafts in non-obese diabetic mice. *Diabetologia* **43**, 474-480 (2000).
32. Isaac, J.R., *et al.* Transplantation of neonatal porcine islets and sertoli cells into nonimmunosuppressed nonhuman primates. *Transplant Proc* **37**, 487-488 (2005).

33. Wang, D.Z., *et al.* Xenotransplantation of neonatal porcine islets and Sertoli cells into nonimmunosuppressed streptozotocin-induced diabetic rats. *Transplant Proc* **37**, 470-471 (2005).
34. Kehrl, J.H., *et al.* Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J Exp Med* **163**, 1037-1050 (1986).
35. Das, L. & Levine, A.D. TGF-beta inhibits IL-2 production and promotes cell cycle arrest in TCR-activated effector/memory T cells in the presence of sustained TCR signal transduction. *J Immunol* **180**, 1490-1498 (2008).
36. Chen, W. & Wahl, S.M. TGF-beta: the missing link in CD4+CD25+ regulatory T cell-mediated immunosuppression. *Cytokine Growth Factor Rev* **14**, 85-89 (2003).
37. Li, M.O., Wan, Y.Y., Sanjabi, S., Robertson, A.K. & Flavell, R.A. Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol* **24**, 99-146 (2006).
38. Fu, S., *et al.* TGF-beta induces Foxp3 + T-regulatory cells from CD4 + CD25 - precursors. *Am J Transplant* **4**, 1614-1627 (2004).
39. Griffith, T.S., Brunner, T., Fletcher, S.M., Green, D.R. & Ferguson, T.A. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* **270**, 1189-1192 (1995).
40. Alderson, M.R., *et al.* Fas ligand mediates activation-induced cell death in human T lymphocytes. *J Exp Med* **181**, 71-77 (1995).
41. Dufour, J.M., Rajotte, R.V., Seeberger, K., Kin, T. & Korbutt, G.S. Long-term survival of neonatal porcine Sertoli cells in non-immunosuppressed rats. *Xenotransplantation* **10**, 577-586 (2003).
42. Dufour, J.M., *et al.* Comparison of successful and unsuccessful islet/Sertoli cell cotransplant grafts in streptozotocin-induced diabetic mice. *Cell Transplant* **16**, 1029-1038 (2008).
43. Dal Secco, V., Riccioli, A., Padula, F., Ziparo, E. & Filippini, A. Mouse Sertoli cells display phenotypical and functional traits of antigen-presenting cells in response to interferon gamma. *Biol Reprod* **78**, 234-242 (2008).
44. Nasr, I.W., *et al.* Testicular immune privilege promotes transplantation tolerance by altering the balance between memory and regulatory T cells. *J Immunol* **174**, 6161-6168 (2005).
45. Roy, M., *et al.* Studies on the interdependence of gp39 and B7 expression and function during antigen-specific immune responses. *Eur J Immunol* **25**, 596-603 (1995).
46. Banchereau, J., de Paoli, P., Valle, A., Garcia, E. & Rousset, F. Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. *Science* **251**, 70-72 (1991).
47. Korthauer, U., *et al.* Defective expression of T-cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature* **361**, 539-541 (1993).

CHAPTER 3

GENERAL DISCUSSION AND CONCLUSIONS

3.1 GENERAL DISCUSSION

The discovery of insulin in 1921 was one of the greatest medical discoveries of all times and has changed the fate of millions who have suffered from diabetes [1]. That being said, type 1 DM remains a chronic disease which necessitates both constant monitoring of blood glucose levels, as well as life-long exogenous insulin therapy. Though there have been immense improvements in the available insulin therapies and insulin pumps, *in lieu* of the multiple factors that cause fluctuations in glycemic levels throughout the day, insulin therapies remain insufficient in maintaining physiological gylcemic levels. This tight regulation of blood glucose levels has been shown to be extremely important, as it prevents both the onset and progression of devastating secondary complications associated with diabetes such as nephropathy, neuropathy, and retinopathy [2]. While a more strict control of blood glucose levels can be attained with intensive insulin therapy, the occurrence of hypoglycemic episodes is greatly increased in patients undergoing such therapy [2]. These hypoglycemic episodes are a major concern as they can lead to severe outcomes such as coma, seizures, or death. Intensive therapy thus becomes even more unrealistic for brittle type 1 diabetic patients who have high glycemic lability and an increased susceptibility towards episodic

hypoglycemia. As such, a more physiological approach to regulating blood glucose levels is imperative to ensure a long and healthy life for patients suffering from this disease.

A number of strategies are currently being investigated in order to achieve this physiological regulation including: i) islet transplantation [3,4]; ii) pancreas transplantation [5-7]; iii) stem cell differentiation [8-10]; and β cell regeneration [10-12]. With the success of the Edmonton Protocol in 2000, islet transplantation has emerged as a realistic treatment modality for patients suffering from type 1 diabetes [3]. In the original Edmonton Protocol study, 7/7 patients that received islet transplants achieved and maintained normoglycemia for at least one year post-transplantation. Islet transplantation was also shown to considerably improve glucose control and alleviated problematic hypoglycemia [4]. While islet transplantation shows great promise, it is currently available to a very select subset of patients, namely brittle type 1 diabetics, due to the toxicity of the immunosuppressive regime. Even more, the limited availability of pancreases from cadaveric donors greatly limits the widespread application of this therapy. As such, safe and effective anti-rejection strategies as well as an adequate alternative source of transplantable islets will need to be found to facilitate the widespread use of this therapy.

Thus, in our study, we wanted to determine the efficacy of short-term administration of anti-LFA-1, anti-CD154, or anti-CD45RB mAb in preventing discordant islet xenograft rejection when NPI are co-transplanted with SC.

Conceptually, this combination of treatment modalities would seemingly have a synergistic effect for multiple reasons. For one, both strategies have been shown to affect $CD4^+$ T cell activity which is imperative in discordant islet xenograft rejection [13]. Each of the monoclonal antibodies being tested have demonstrated a capacity to interfere with T cell activation and their ability to mount an immune response while SC have shown an ability to interfere with $CD4^+$ T cells by inhibiting IL-2 production and T cell expansion [14,15], depleting activated lymphocytes via FasL secretion [16], and regulating immune reactivity with the secretion of TGF-β [17].

Monoclonal antibody therapies may also provide inhibition of humoral responses [18,19], presumably via interfering with helper T cell activation and subsequently B cell help. This combined with the complement inhibition provided by clusterin , a major secretion of SC, may provide a synergistic effect towards the prevention of antibody mediated destruction [20]. The mAb therapies used in this study have also demonstrated an ability to generate or facilitate protection of islet xenografts by regulatory T cells [21]. This combined with the foxp3 inducing properties of TGF-β may provide a coordinated effort in the induction of T regulatory cells that can prevent graft rejection. TGF-β may result in immune modulation of islet tissue as well, thus reducing overall graft immunogenicity and susceptibility towards rejection [22].

As such, there are a plethora of mechanisms that may be involved to either work coordinately to enhance a protective effect, or individually to inhibit non-overlapping processes in xenograft rejection, ultimately leading to a synergistic

effect and prolongation of graft survival. Indeed in our study, we have demonstrated that the combination of SC with mAb therapy is considerably more effective than when NPI are transplanted alone and given mAb therapy. Here we found that 7/7 (100%) mice treated with anti-LFA-1 mAb, 7/8 (88%) mice treated with anti-CD154 mAb, and 4/9 (44%) mice treated with anti-CD45RB mAb that were transplanted with NPI and SC achieved and maintained normoglycemia long-term. In comparison, none of the untreated control mice transplanted with NPI and SC (0/8) achieved normoglycemia. Histological analysis of the graft tissue of mice that achieved long-term graft survival shows the presence of insulin, vimentin, and MIS positive cells, demonstrating the presence of both β cells and SC in the grafts at the endpoint of the study. Histological assessment of the grafts also revealed mononuclear cell infiltrates within all the treatment groups which consisted primarily of $CD4^+$ T cells and $CD19^+$ B cells, with relatively low numbers of $CD8^+$ T cells and $CD11b^+$ macrophages. Interestingly, $foxp3^+$ cells were also present within the grafts of mice that had prolonged graft survival, indicating a possible function of T regulatory cells in graft protection.

Anti-donor reactivity was indirectly measured by the relative production of anti-porcine antibodies in sera of recipient mice. Here it was shown that mAb therapy effectively reduced anti-donor antibody production in mice that had long-term graft survival, whereas high levels of antibody production were seen in mice that rejected their grafts. Two major exceptions to this were mice treated with anti-CD154 mAb and mice treated with anti-LFA-1 mAb that were transplanted with NPI and SC. In the former, antibody levels were low regardless of rejection,

whereas in the later, considerable variability in antibody production existed and seemed independent of graft rejection. This variability with the anti-LFA-1 mAb treatment may have been because some of these mice were in the process of rejection and so the antibody production was an indicator of an increased antigen specific reactivity towards the islet graft. It may also be that anti-LFA-1 mAb was not sufficient to suppress the increased humoral mediated response elicited due to the additional antigens present from transplanting the SC with the NPI. However, this humoral response seemed to be insufficient, at least for the first 100 days, in causing graft rejection *in lieu* of the 100% graft survival seen in this treatment group. Thus, even though we were able to achieve long-term graft survival in all recipients treated with anti-LFA-1 mAb and SC, the longevity of graft survival beyond 100 days will need to be examined to determine whether or not these mice are actually in the process of rejecting their graft. Future studies will also need to examine the reason for the increased humoral reactivity and determine if the elicited humoral response is an important indicator or contributor towards the destruction of the islets.

While we have been able to show superior graft protection using this combination of strategies, there are a number of other questions that remain. Four major areas that will need further investigation are: *i)* determining the mechanisms involved in the graft protection induced with this combination of strategies; *ii)* determining the type of protection induced; *iii)* optimizing the delivery of this combination of strategies; and *iv)* determining the efficacy and

safety of these treatments in more appropriate models (i.e. large animal and autoimmune transplant models).

At present, there is no clear understanding of the mechanisms involved in the protection we see with this combination of therapies. That is, we cannot say with confidence that the protection induced is actually mediated by the mechanisms we proposed above, as there may also be other pathways and molecules involved. We also do not know the importance of each of the above mentioned secreted products of SC, i.e. the contribution or requirement of TGF-β, FasL, clusterin, serpina3n, EGF, IGF-1, IGF-2, among others. If we find that only one of these molecules is truly important, perhaps this molecule could be used more appropriately and be optimized to maximize the efficacy and safety of this treatment. Even more, if the changes that result in the immune system due to SC transplantation or anti-LFA-1 mAb can be identified, more appropriate means of inducing these changes could assist in improving this anti-rejection strategy. That is, even though this treatment was able to invoke long-term graft survival, neither the response to glucose challenge nor the survival of the islets past 100 days were tested, and so there is most definitely room for improvement. It is also possible that fewer islets may be needed for transplantation if the therapy could be optimized further, if for example more islets could be spared from destruction or if under certain conditions SC could improve the function and maturation of the islets in our model. Thus, an understanding of the mechanism of protection may allow us to further improve this treatment.

In the same respect, with the current information we have, we cannot with confidence determine why anti-LFA-1 mAb was the most effective mAb therapy or through what mechanisms it was able to enhance xenograft survival. For example, we saw a heavy infiltrate within our islet grafts with anti-LFA-1 mAb treatment, suggesting that inhibition of lymphocyte migration and/or ignorance cannot fully explain its protective effects. That being said, we also showed that foxp3$^+$ cells were present in the graft site of mice treated with mAb therapy and SC, and so it is possible that the mechanism of protection induced by these treatment strategies is via the generation of antigen-specific T regulatory cells. The role of T regulatory cells can be further analyzed by: *i)* looking for increases in regulatory cytokines within the grafts of protected versus rejected mice; *ii)* analyzing spleen and lymph node cells for increases in T regulatory cell markers (i.e. CD4$^+$CD25$^+$foxp3$^+$, CD4$^+$CD25$^+$GITR$^+$, and CD4$^+$CD25$^+$PD1$^+$ cells); *iii)* depleting T regulatory cells using anti-CD25 mAb to determine if protection is abolished in the absence of these cells; and *iv)* looking for triple positive CD4$^+$CD25$^+$foxp3$^+$ cells within the grafts of protected mice (more accurate phenotype of T regulatory cells compared to foxp3 alone). Future studies can also be done to determine if there are any changes in systemic levels of various cytokines of protected mice versus naïve and/or rejected mice. This may assist in determining if particular cytokines are involved in the protection seen with this combination of treatments. Systemic changes in the immune cell composition (i.e. CD4$^+$ cells, CD8$^+$ cells, CD19$^+$ cells, CD25$^+$ cells, among others) and/or expression of co-inhibitory molecules (i.e. PD1, BTLA, and CTLA-4) would also

help in discerning whether certain populations of cells or molecules are important in the protection induced with this combination of treatments. Therefore, future studies will be required to elucidate the actual mode and mechanism of protection induced by these treatments.

It will also be important to know whether this combination of strategies leads to the development of tolerance or just graft prolongation. The difference here is that antigen specific tolerance would mean that the immune system is no longer reactive to the islet graft whereas graft prolongation just postpones the rejection process. Thus, if tolerance was induced, the graft would remain protected indefinitely without the requirement for additional therapy. If it can be determined that a state of tolerance is induced (for example through second party transplants and adoptive transfer experiments), we will have to investigate the specificity of this tolerance. For example, has our therapy resulted in a donor-specific, islet-specific, or pig-specific unresponsiveness? The importance of determining this is that a second or third transplant is generally required in clinical islet transplantation. Thus, would the protection we induced with this combination of treatments be extended to a new islet graft, or would there be a requirement for a second round of mAb and SC therapy. If however it is determined that this combination merely leads to graft prolongation, maintenance therapy and multiple transplants will be required to sustain insulin independence in the islet transplant recipients , which would again limit the clinical applicability of this therapy.

Optimization of this combination of strategies is also an area that requires further investigation. For example, the ratio of islets and SC that are transplanted has been shown to have an important role in an autoimmune transplant model [23], and thus optimization of this parameter may further enhance the protection we see. Even more, the current thesis investigated the protective properties of neonatal porcine SC. Seeing that SC mediated protection is likely most important after puberty (when gametes are being produced), it is likely that post-pubertal SC would be more protective. Thus, the maturation and potential differences in function between mature and immature SC will need to be considered further to determine which source has the greatest potential to protect islets. In our study, the SC donor was different from the NPI donor as well. Therefore, future studies can be conducted to determine if the donor of the SC affects the protective capacity of these cells. We also saw that the islets that were co-transplanted with SC were fragmented, suggesting that SC may have deleterious effects on islets as well. This may be due to the overgrowth of SC leading to a competition for space and nutrients. It may also be due to the secretion of FasL by SC which may directly kill Fas expressing islets, or enhance neutrophilic infiltrate as shown previously [23,24]. Even more, SC have been shown to produce IL-1 which can enhance Fas expression, further promoting β cell death [25,26]. Thus, if the deleterious effects of SC can be identified and subsequently minimized or eliminated, the effectiveness of this strategy may be further enhanced. To reiterate, though 100% of the mice that received islets and SC with anti-LFA-1 mAb achieved long-term graft survival, teasing out the negative effects of these

therapies may further increase the longevity of graft survival, may decrease the number of islets required for transplantation, may improve islet graft function (i.e. response to glucose), or may decrease the required dose of anti-LFA-1 mAb which would enhance the safety and/or efficacy of this strategy.

Another important issue to consider with the strategies tested is their safety and efficacy in more appropriate islet transplant models. While anti-LFA-1 mAb has been tested clinically and is being used for the treatment of psoriases, its safety and efficacy in islet transplantation has yet to be determined. Further, transplantation of testicular cells (in this case SC) into both males and females could potentially cause irregularities in hormone balance in the body as SC are responsive to follicular stimulating hormone and testosterone. Thus, a thorough investigation of the side effects of transplanting SC in a large animal model would provide a better understanding of the applicability of this therapy. It will also be advantageous to test this strategy in an autoimmune model of type 1 diabetes so that the efficacy of this combination can be determined in a model more relevant to human type 1 diabetic patients.

Overall, our study demonstrates that transient administration of anti-LFA-1 mAb or anti-CD154 mAb is highly efficacious in prolonging NPI xenograft survival when islets are co-transplanted with SC. Thus, further experiments will need to be done to analyze the mechanism of protection induced by this combination of strategies, to investigate the stability and/or robustness of this

protection, to optimize the delivery of these treatments, and to determine its safety and efficacy in other models.

3.2 CONCLUSION

Islet transplantation has emerged as a viable treatment strategy for severe cases of type 1 diabetes, however it holds the potential to re-establish precise glucose control to all type 1 diabetic patients. A return to a life without worry about glucose control and insulin injections, life-style and dietary restrictions, the predisposition towards the development of secondary complications, and the fear of hypoglycemia provides impetus for the improvement and widespread application of this therapy.

Two major barriers however, have limited the widespread use of islet transplantation: *i)* the shortage of available islets for transplantation; and *ii)* the requirement for chronic immunosuppression to prevent graft rejection. To remedy the shortage of donor islets, xenotransplantation of NPI has emerged as a viable alternative source of transplantable tissue pending confirmation of the safety of this source and the establishment of safe and effective ant-rejection strategies.

To succeed clinically, strategies that can prevent graft rejection in a manner that is safe for all patients, including young type 1 diabetic patients, will need to be developed. Here we have demonstrated that the combination of anti-LFA-1 or anti-CD154 mAb therapy combined with co-transplantation of NPI with

SC is highly effective in preventing the rejection of NPI xenografts. Future studies will therefore need to be done to delve more into the mechanism of protection and determine the stability of this protection. It is our hope that the information presented in this manuscript will provide further insights on the process of islet xenograft rejection and contribute to the body of knowledge that will hopefully translate into therapies that will improve the health and quality of life of all patients suffering from this disease.

3.3 REFERENCES

1. Pickup, J.C. & Williams, G. *Textbook of diabetes*, (Blackwell Science, Malden, Mass., 2003).
2. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. *N Engl J Med* **329**, 977-986 (1993).
3. Shapiro, A.M.*, et al.* Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* **343**, 230-238 (2000).
4. Ryan, E.A.*, et al.* Five-year follow-up after clinical islet transplantation. *Diabetes* **54**, 2060-2069 (2005).
5. Humar, A.*, et al.* Decreased surgical risks of pancreas transplantation in the modern era. *Ann Surg* **231**, 269-275 (2000).
6. Tyden, G.*, et al.* Improved survival in patients with insulin-dependent diabetes mellitus and end-stage diabetic nephropathy 10 years after combined pancreas and kidney transplantation. *Transplantation* **67**, 645-648 (1999).
7. Sutherland, D.E.*, et al.* Lessons learned from more than 1,000 pancreas transplants at a single institution. *Ann Surg* **233**, 463-501 (2001).
8. Peck, A.B., Cornelius, J.G., Chaudhari, M., Shatz, D. & Ramiya, V.K. Use of in vitro-generated, stem cell-derived islets to cure type 1 diabetes: how close are we? *Ann N Y Acad Sci* **958**, 59-68 (2002).
9. Street, C.N., Rajotte, R.V. & Korbutt, G.S. Stem cells: a promising source of pancreatic islets for transplantation in type 1 diabetes. *Curr Top Dev Biol* **58**, 111-136 (2003).
10. Shapiro, A.M.J. & Shaw, J.A.M. *Islet transplantation and beta cell replacement therapy*, (Informa Healthcare, New York, 2007).
11. Trucco, M. Regeneration of the pancreatic beta cell. *J Clin Invest* **115**, 5-12 (2005).
12. Bouwens, L. & Rooman, I. Regulation of pancreatic beta-cell mass. *Physiol Rev* **85**, 1255-1270 (2005).
13. Rayat, G.R.*, et al.* The degree of phylogenetic disparity of islet grafts dictates the reliance on indirect CD4 T-cell antigen recognition for rejection. *Diabetes* **52**, 1433-1440 (2003).
14. De Cesaris, P.*, et al.* Immunosuppressive molecules produced by Sertoli cells cultured in vitro: biological effects on lymphocytes. *Biochem Biophys Res Commun* **186**, 1639-1646 (1992).
15. Selawry, H.P., Kotb, M., Herrod, H.G. & Lu, Z.N. Production of a factor, or factors, suppressing IL-2 production and T cell proliferation by Sertoli cell-enriched preparations. A potential role for islet transplantation in an immunologically privileged site. *Transplantation* **52**, 846-850 (1991).
16. Bellgrau, D.*, et al.* A role for CD95 ligand in preventing graft rejection. *Nature* **377**, 630-632 (1995).

17. Suarez-Pinzon, W., *et al.* Testicular sertoli cells protect islet beta-cells from autoimmune destruction in NOD mice by a transforming growth factor-beta1-dependent mechanism. *Diabetes* **49**, 1810-1818 (2000).
18. Tredget, E.B., Arefanian, H., Gill, R.G., Rajotte, R.V. & Rayat, G.R. Monotherapy with anti-LFA-1 monoclonal antibody promotes long-term survival of rat islet xenografts. *Cell Transplant* **17**, 599-608 (2008).
19. Rayat, G.R. & Gill, R.G. Indefinite survival of neonatal porcine islet xenografts by simultaneous targeting of LFA-1 and CD154 or CD45RB. *Diabetes* **54**, 443-451 (2005).
20. Dufour, J.M., Hamilton, M., Rajotte, R.V. & Korbutt, G.S. Neonatal porcine Sertoli cells inhibit human natural antibody-mediated lysis. *Biol Reprod* **72**, 1224-1231 (2005).
21. Arefanian, H., Tredget, EB., Rajotte, RV., Gill, RG., Korbutt, GS., Rayat, GR. Short-Term Administrations of a Combination of Anti-LFA-1 and Anti-CD154 Monoclonal Antibodies Induces Tolerance to Neonatal Porcine Islet Xenografts. *Diabetes* **In press**(2009).
22. Carel, J.C., Schreiber, R.D., Falqui, L. & Lacy, P.E. Transforming growth factor beta decreases the immunogenicity of rat islet xenografts (rat to mouse) and prevents rejection in association with treatment of the recipient with a monoclonal antibody to interferon gamma. *Proc Natl Acad Sci U S A* **87**, 1591-1595 (1990).
23. Korbutt, G.S., Suarez-Pinzon, W.L., Power, R.F., Rajotte, R.V. & Rabinovitch, A. Testicular Sertoli cells exert both protective and destructive effects on syngeneic islet grafts in non-obese diabetic mice. *Diabetologia* **43**, 474-480 (2000).
24. Kang, S.M., *et al.* Fas ligand expression in islets of Langerhans does not confer immune privilege and instead targets them for rapid destruction. *Nat Med* **3**, 738-743 (1997).
25. Hedger, M.P. & Meinhardt, A. Cytokines and the immune-testicular axis. *J Reprod Immunol* **58**, 1-26 (2003).
26. Guazzone, V.A., Jacobo, P., Theas, M.S. & Lustig, L. Cytokines and chemokines in testicular inflammation: A brief review. *Microsc Res Tech* (2009).