University of Alberta

Rapamycin and Anti-LFA-1 Monoclonal Antibody Prevent the Rejection of Rat Islet Xenografts and Induce Tolerance in BALB/c Mice

by

Eric Britt Tredget

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

Masters of Science
in
Experimental Surgery

Department of Surgery

Edmonton, Alberta
Fall 2006

Your file Votre référence
ISBN: 978-0-494-22389-5
Our file Notre référence
ISBN: 978-0-494-22389-5

ABSTRACT

Despite recent advancements in islet transplantation, this therapy is not available to the vast majority of patients with type 1 diabetes mellitus (T1DM) because of the shortage of donor islets and the toxicity of the immunosuppressive regimen. Xenogeneic islet transplantation holds promise for clinical transplantation because of the potentially unlimited supply of islets. However, xenogeneic islets are subject to a robust immune response. This has prompted investigation of new ways of preventing xenogeneic islet rejection that can be given short term, abrogating the toxicity associated with chronic immunosuppression. New evidence suggests that monoclonal antibodies specific for immune cell surface molecules could be employed in the prevention of islet graft rejection without the need for continuous administration. In this study we determined whether short-term administration of the immunosuppressive drug, rapamycin, combined with anti-LFA-1 monoclonal antibody would promote long-term survival of rat islet xenografts in mice and induce tolerance to these islet grafts.

Greater than 96% of mice that received the combination therapy of rapamycin and anti-LFA-1 maintained their islet grafts for greater than 100 days. In addition, a subset of mice, were re-challenged with the same rat islet xenograft; all recipients maintained long-term islet graft function with no administration of anti-rejection drugs whatsoever.

The combination of rapamycin and anti-LFA-1 in concordant xenogeneic islet transplantation appears extremely efficacious at inducing permanent islet graft survival and tolerance, and may form a critical part of future regimens used to prevent the rejection of xenogeneic islets in clinical islet transplantation.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor, Dr. Gina R. Rayat for showing confidence in me, and allowing me to pursue my love of science under her guidance. She is the most wonderful supervisor one could ask for, and has become a respected mentor and friend who has always had time to listen and offer helpful advice, whether it be for my professional or personal life. Thank you so much for everything Gina.

I would also like to thank Dr. Ray V. Rajotte for the wonderful guidance and support he has given me over these past years. Dr. Rajotte's dedication to his students is remarkable, and I hold him in the highest respect. I feel truly privileged to have had the opportunity to work with him. Dr. Gregory S. Korbutt has always been a friendly face in lab, and I would like to thank him for the advice and support he has given me. I would also like to thank Dr. Korbutt for sharing his lab and equipment with us during our lab's infancy. Special thanks go to Dr. Catherine J. Field for her insight, comments and critical review of this manuscript.

I would like to extend a special thanks to members of our lab. Hossein Arefanian has become a wonderful friend of mine, one who has opened my eyes to a different part of the world, and has made me realize that our cultures are not as different as some may think. He always been there to lend a helping hand, and I wish he and his family all the best in the future. Leilani Langer is another wonderful and trusted friend of mine who has always been there to lend an ear or share a laugh. I wish her a lifetime of happiness, whatever road she chooses. Thank you to Dereck Mok and Tsune Kobayashi for their

friendship, advice, and for the laughs, whether they were in the lab or on the golf course! Special thanks go to our wonderful summer students: Graeme Armstrong, Raissa Petriw, Matt Sadler and Stephanie Wilkes for all their hard work.

Thank you to the staff of SMRI, especially Rosemarie Henley, Margaret Henley and Colleen Ruptash who have always gone above and beyond for me. Deb Dixon has been an invaluable part of my project and a wonderful teacher and mouse encyclopedia. I owe Dawne Colwell a special thanks for her wonderful artistry, which have been instrumental in my posters, presentations and this manuscript.

A special thanks to all of the members of the Korbutt Lab, especially Karen Seeberger, Lynette Elder, and Alanna Eshpeter and James Lyon. I am also very grateful to the Alberta Diabetes Foundation, the Department of Surgery, the Faculty of Graduate Studies and Research, and the Graduate Students' Association for their financial support.

Finally, I would like to thank my family, and the Fraser's for their unyielding support and love, without which, the completion of this thesis would not have been possible. Lastly, my deepest love and thanks go to my best friend, Jill Anne Fraser, who has been my support and inspiration for everything in life.

This text is dedicated to Jill Anne Fraser and to my mother Jean and my father Ted for the tremendous support and love they have always given me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α1,3GT	α1,3 galactosyltransferase
ABC	avidin-biotin complex
ADCC	antibody-dependent cell-mediated cytotoxicity
ADP	adenosine diphosphate
ANOVA	analysis of variance
APCs	antigen-presenting cells
ATP	adenosine triphosphate
AVR	acute vascular rejection
B6	C57BL/6
BB rat	bio-breeding rat
BSA	bovine serum albumin
CD	cluster of differentiation
CK	cytokeratins
CTL	cytotoxic T lymphocytes
CTLA-4	cytotoxic T lymphocyte antigen-4
ConA	concanavalin A
DAB	3,3-diaminobenzidinetetrahydrochloride
DAF	decay accelerating factor
DC	dendritic cell
DXR	delayed xenograft rejection
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EMEM	Earl's minimum essential medium
FACS	fluorescence activated cell sorter
FasL	Fas ligand
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FoxP3	forkhead box P3
GAD	glutamic acid decarboxylase
Galα(1,3)Gal	galactoseα(1,3)galactose
HAR	hyperacute rejection
HBSS	Hank's balanced salt solution
H/E	Hematoxylin/Eosin
HI	heat-inactivated
HLA	human leukocyte antigen
HP	horseradish peroxidase
IAA	insulin autoantibodies
IBMIR	instant blood-mediated inflammatory reaction
IL-1	interleukin-1
IFN-γ	interferon-γ
Ig	immunoglobulin
i.p.	intraperitoneal
MHC	major histocompatibility complex

NK	natural killer
NOD	non-obese diabetic
NPI	neonatal porcine islets
OGTT	oral glucose tolerance test
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PDX-1	pancreatic and duodenal homeobox gene-1
PERV	porcine endogenous retrovirus
PG	plasma glucose
Pp	polypeptide
P/S	penicillin/streptomycin
SEM	standard error of mean
SLA	swine leukocyte antigen
TCR	T cell receptor
TGF-β	transforming growth factor-β
T_H1	T helper cell 1
T_H2	T helper cell 2
TNF-α	tumor necrosis factor-α
WHO	World Health Organization
XNA	xenoreactive natural antibodies

CHAPTER 1

GENERAL INTRODUCTION

1.1 DIABETES MELLITUS

Diabetes mellitus is a chronic metabolic disease, which is characterized by high blood glucose levels or hyperglycemia. Hyperglycemia in these patients is the result of either a lack of insulin secretion, an insufficient level of insulin secretion or the secreted insulin not being as effective as it should be (1). Diabetes is a disease which is on the rise. At present, greater than 171 million people have diabetes. Between 1985 and 1995 the incidence of diabetes more than quadrupled, and in the next 20 years the World Health Organization (WHO) estimates that the number of people with diabetes is expected to double.

1.1.1 TYPE 1 DIABETES MELLITUS

Type I diabetes mellitus (T1DM) is an autoimmune disease that typically manifests in childhood between the ages of 10-14, however, recent trends suggest that the age of onset of T1DM is decreasing (1). The autoimmune response in T1DM is targeted towards specialized insulin producing cells called β cells located in the islets of Langerhans within the pancreas. The autoimmune attack results in the destruction of the beta cells, and as a consequence, the insulin producing ability of the pancreas is destroyed. T1DM is characterized by an absolute lack of insulin secretion and treatment therefore centers around the replacement of insulin (1-4). The most recent clinical definition as described by the WHO, of a patient with diabetes mellitus is a casual plasma glucose (PG) level of 11.1 mmol/L or more, or, a fasting PG level of 7.1mmol/L or more,

and/or, a 2 hour post-glucose challenge PG level of 11.1mmol/L or more (1). While T1DM comprises only ~10% of patients with diabetes mellitus, an estimated 17 million people are afflicted with T1DM with an increasing incidence of ~3% each year in most countries (1;2). The Canadian Diabetes Association estimates that in Canada, T1DM affects more than 225,000 people and is associated with escalating costs to the Canadian health care system.

1.1.2 HISTORY OF T1DM

The term "diabetes" is derived from the Greek word meaning siphon and was first coined by Aretaeus of Cappadocia in the 2nd century AD. While the name "diabetes" was not used until the 2nd century, the polyuric conditions of the disease were known to the Egyptians, and were published in Ebers papyrus (written between 300-1500 BC). Two Hindu physicians Charak and Sushrut were believed to be the first to recognize the sweetness of diabetic urine and noted sometime around ~ 450BC that the urine tasted sweet and that ants congregated around it. The term "mellitus" was first used by John Rollo in 1809 and is derived from the Latin and Greek words meaning honey (1).

Before the discovery of insulin, patients with T1DM experienced dangerous fluctuations in their blood glucose values, and a greatly shortened life expectancy. It was not until 1921 that insulin was discovered in Toronto by a team of researchers: Frederick Banting, Charles Best, James Collip and J.J.R Macleod. The discovery of insulin revolutionized the treatment of T1DM and it transformed what was at the time, an acutely fatal illness, into a chronic disease (1;5;6). Despite this leap forward in the treatment of T1DM, diabetes presently accounts for 40% of all cases of end-stage renal disease, 25%

of all cardiac surgery, and 50% of all non-trauma associated amputations. In addition, patients with chronic T1DM are at risk of foot ulceration, retinopathy, neuropathy, and nephropathy, highlighting the need for better treatment alternatives for people with T1DM (3;7-16).

1.1.3 EPIDEMIOLOGY AND ETIOLOGY OF T1DM

Interestingly, the distribution of T1DM patients is not uniform throughout the world. There are concentrated regions in which the incidences of new cases of T1DM is extremely high including: Finland, Sardinia (Italy), Sweden, Prince Edward Island, and Alberta. In addition, marked variations in the incidence of diabetes can be detected within countries. For example, the incidence of T1DM is 3-5 times higher in Sardinia when compared to mainland Italy. These findings suggest strong evidence for the roles of genetic and environmental factors in the etiology of T1DM (1).

The autoimmune response in T1DM is targeted towards specialized insulin producing cells called beta cells, which are clustered in the islets of Langerhans within the pancreas. It is hypothesized that an environmental trigger and an underlying genetic susceptibility initiates an autoimmune response, resulting in the progressive destruction of the β cells. β cell auto-antigens, dendritic cells, macrophages, T cells and B cells are known to be involved in the pathogenesis of autoimmune diabetes (17;18). B cells are believed to play a role in the initial stages of disease progression as antigen presenting cells (APCs), and then later on via the secretion of auto-antibodies. Animal models of T1DM have demonstrated that T cells play a critical role as effector cells which directly mediate β cell killing (17;18). Cytokines secreted by macrophages and helper T cells (T_H

cells), are believed to polarize the immune response to either a T_H1 or T_H2 phenotype. β cells are believed to be destroyed by apoptosis via Fas-Fas ligand and by granzymes and perforin released from cytotoxic T cells (T_C cells)(17;18). The autoimmune attack results in the elimination of the β cells, destroying the insulin producing ability of the pancreas (2;4;19). While the specific antigens targeted by the immune system are still debated, the enzyme glutamic acid decarboxylase (GAD) and insulin appear to be the most likely auto-antigens. This is based on studies which have found that the presence of GAD antibodies and insulin auto-antibodies (IAA) is associated with an increased risk of developing T1DM (3;4;20).

1.1.3.1 Genetic Factors

It has been demonstrated that the susceptibility to the development of T1DM is most significantly influenced by human leukocyte antigen (HLA) class II genes (21). There have been several mechanisms put forth to explain the functional differences between various HLA class II molecules, and why some haplotypes appear to be protective and others are associated with an increased risk of T1DM. One theory is that protective HLA molecules may bind strongly to self antigens to form stable complexes in the thymus consequently, facilitating the effective deletion of potentially auto-reactive T cells. Conversely, if an unstable complex is formed between self antigens and diabetic HLA haplotypes, it may permit the auto-reactive T cells to proliferate and allow these T cells to react with self antigens. An alternative theory is that diabetic susceptible HLA molecules may bind to self antigens and activate auto-reactive T cells, whereas protective HLA molecules bind strongly to these antigens and compete with diabetic HLA

haplotypes for binding sites. The third possibility is that HLA molecules, whether they be associated with T1DM or in the protection from T1DM, may interact differently with the T cell receptor of auto-reactive T cells and may affect whether the T cell goes on to be proinflammatory, regulatory or whether the T cell is activated or not (3;4;20-23). The strongest genetic link in T1DM is found at the IDDM1 locus, which encompasses the human leukocyte antigen (HLA) gene cluster. While the HLA-DQ6 genotype appears to protect against the development of T1DM, two HLA class II haplotypes in particular, have been associated with an increased risk of developing T1DM, HLA-DR3 and HLA-DR4. At least one of the HLA-DR3 or HLA-DR4 genotypes is present in 95% of Caucasian patients with T1DM. Specifically, possession of the HLA-DRB1*03.DQ2 and HLA-DRB1*04.DQ8 predisposes individuals to T1DM, however the presence of these HLA haplotypes in no way guarantees the eventual onset of T1DM (24-27). It is clear that environmental aspects along with genetic factors play a role in the development of T1DM.

1.1.3.2 Environmental Factors

The strongest argument for the role of environmental factors in the development of T1DM comes from studies on monozygotic twins. It has been found that if one twin has been diagnosed with T1DM, the chance that the other genetically identical twin will also develop T1DM is no more than 40% (1;2). Environmental factors such as viral infections and certain dietary components are believed to contribute to the development of T1DM.

Evidence that supports the role of a viral infection as a contributor to the onset of T1DM includes seasonal variation and clustering of new cases of T1DM. In addition, some recently diagnosed T1DM patients demonstrate signs of viral infection and there have been many cases of new onset T1DM occurring soon after or during viral infections (28). Many viral infections have been potentially identified in the etiology of T1DM, including, but not limited to, rubella, coxsackie, mumps, and cytomegalovirus (CMV) infections (28). Rubella infections that occur *in utero* typically result in the development of T1DM in 12-20% of individuals (29). Rubella infections are believed to provoke T1DM through molecular mimicry of β cell antigens or through alteration of β cell antigens and subsequent presentation on the cell surface leading to activation of the host's immune system (30). Coxsackie infections are thought to induce T1DM through eliciting an immune response raised against the virus but which also cross-reacts with β cell specific antigens (31). Mumps virus infections are thought to contribute to the onset of diabetes via production of interleukins and by inducing increased expression of HLA class I and II by the β cell (32). Like rubella infections, CMV infections are believed to aid in the induction of diabetes through molecular mimicry of β cell specific antigens (33).

Aside from viral infections, other possible environmental factors, which could contribute to the onset of T1DM have been implicated and include: insufficient/lack of breast feeding, exposure to nitrosamines, and exposure to wheat proteins. Insufficient or a lack of breast feeding has been the most extensively studied dietary factor contributing to T1DM (34). While a clear mechanism as to how breast feeding prevents the induction of T1DM has yet to be elucidated, several studies have linked early exposure to cow's

milk, which commonly forms the basis of many infant formulas, to the development of T1DM (35-37). One of the leading hypotheses is that exposure to cow's milk which contains low levels of insulin compromises tolerance to insulin in the host. Infants which were fed cow milk-based infant formula, instead of human milk demonstrated an increase in the level of IgG antibodies during their first 9 months (38-40). Interestingly, human milk has been found to contain approximately 4 times the amount of insulin that cow's milk does, prompting suggestions that human insulin should be added to infant formulas to potentially induce oral tolerance to insulin and thus help prevent the onset of diabetes (41;42). Nitrosamine exposure has also been linked to T1DM. Nitrate and nitrites are commonly found in foods and can readily react with amines and amides to produce N-nitroso compounds. This is of particular importance because the chemical streptozotocin (STZ), which is used to induce diabetes in experimental animals is an N-nitroso compound. Therefore, foods which are high in nitrates and nitrites such as smoked meats are hypothesized to contribute to the onset of T1DM by damaging β cells in a mechanism similar to STZ (43;44). Wheat proteins have been found to cause aberrant immune responses in susceptible individuals. A specific example of this is Celiac's disease, where wheat peptides cause inappropriate immune stimulation and result in the production of IgE and promote immune-mediated damage to the gut. In the T1DM animal model, the BB rat, wheat gluten is a potent diabetic antigen (45;46). While there is little evidence linking wheat proteins to the development of T1DM, it has been found that 5-10% of people with T1DM have Celiac's disease, which is a rate 17-33 times higher that of the general population (47-49) suggesting a partial association of wheat proteins in the etiology of T1DM.

1.1.4 ANATOMY AND PHYSIOLOGY OF THE PANCREAS IN T1DM

Located in the abdominal region, the pancreas is one of several organs that comprises the endocrine system. While the endocrine function of the pancreas is of most importance in T1DM, the pancreas also has an exocrine function, which aids in digestion. The islets of Langerhans are located in scattered clusters within the pancreas and are the secretory component of the endocrine function of the pancreas (50;51). The islets are typically most concentrated in the body and tail of the pancreas (Figure 1-1). The principal hormones secreted by the islets are insulin, glucagon, somatostatin and pancreatic polypeptide. These hormones are secreted by β cells, α cells, δ cells and PP cells respectively (52;53). Insulin and glucagon have opposing effects. Insulin promotes the cellular uptake of glucose by peripheral tissues and the formation of glycogen by the liver and assists in the synthesis of proteins and fat. Glucagon conversely, stimulates the hydrolysis of glycogen by the liver. Somatostatin, secreted by δ cells dampens the effects of insulin and glucagon by suppressing the secretion of both insulin and glucagon. Pancreatic polypeptide is expressed exclusively in the pancreas, however to date, its function remains unknown (1). Islets while serving as the major secretory component of the endocrine function of the pancreas, compose only 1-2% of the entire pancreatic mass in adult mammals. It is widely speculated that only 10-30% of the endocrine cells are necessary for maintaining normoglycemia (54).

In a normal individual, blood glucose levels (BGLs) are maintained in a very strict balance. Carbohydrate oxidation provides approximately 45% of all the energy expended in a person at rest. In the brain however, greater than 99% of the energy

consumed is provided by carbohydrate oxidation. In an individual with T1DM, however virtually no endogenous insulin is present, restricting the effectiveness of glucose as a fuel source. This places increased demand on other sources of energy namely, fat (including ketone bodies) and protein. Prolonged periods of insulin deprivation is associated with an increased production of ketone bodies which can lead to ketoacidosis, an important cause of morbidity and mortality in T1DM (55-57). In addition, the increased demand on protein as an energy source can result in muscle wasting, during periods of prolonged insulin deficiency (58;59).

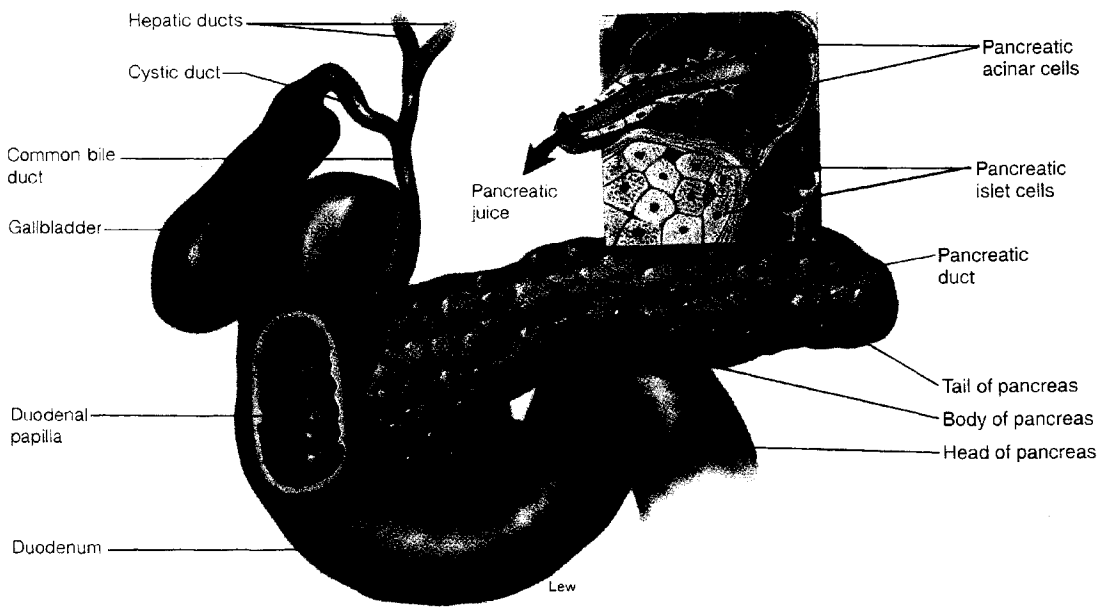


Figure 1-1. The pancreas and associated organs. (Taken from Van de Graff KM, Human Anatomy, 2002, 6th Edition, McGraw-Hill, Toronto).

1.2 NEW APPROACHES FOR THE TREATMENT OF T1DM

Traditionally, the treatment of T1DM has been accomplished through exogenous insulin administration. Various short and long acting formulations have greatly increased the glycemic control of T1DM and dramatically improved the quality of life for these patients. However, without careful blood glucose monitoring and dosage calculation the patient can still experience dangerous fluctuations in blood glucose levels which can have damaging, progressive effects on the body (60). Even in the most conscientious patient, bolus injections of insulin cannot compete with the kind of blood glucose control that is found in people with functional islets, and typically at least some detectable side effects can be found in patients with chronic diabetes (59). Therefore the focus of research has been primarily toward developing the methods and techniques required for the replacement of insulin producing tissue or the regeneration of the insulin producing beta cells.

1.2.1 ISLET TRANSPLANTATION

The concept of transplanting pancreatic tissue has intrigued researchers since 1889 when Von Mering and Minkowski reported that the removal of the canine pancreas rendered the animal unable to maintain a normal blood glucose level and consequently became hyperglycemic (61;62). The practice of transplanting individual islets was not performed until Hellerstrom, using a microdissection technique, isolated rat islets from the pancreas. The yield of islets using this procedure was low and prompted more efficient and effective methods of purification (63). To improve islet yield, Moskalewski used collagenase to enzymatically digest an intact pancreas (64). In 1967, Lacy and

Kostianovsky further refined islet isolation by employing a novel technique which involved distending the pancreas by intraductal infusion of collagenase (65). To purify islets from contaminating exocrine and connective tissue, techniques involving density centrifugation were applied, initially with sucrose. After further refinement of the technique, Ficoll density gradients proved to be a more suitable osmotic environment for the islets and improved islet viability (66). The first reported islet transplantation was performed by Younoszai *et al.* in 1970 (67). The group successfully transplanted rodent islets and observed a brief reduction in hyperglycemia in chemically induced diabetic rats (62). This study was followed shortly by a report from Ballinger and Lacy in 1972, where the group successfully transplanted intact islets into rats (68). While the successful islet isolation technique in rats appeared very promising, when the technique was applied to larger mammals including primates, new problems were encountered. The more compact and fibrous pancreas found especially in humans, proved to be more resilient to digestion and consequently, the preparations produced poor islet yield and viability (69). After many advancements in large mammal islet isolation, it was determined that the yield of islets could be substantially increased if collagenase was introduced directly by ductal injection. This method allowed the collagenase to effectively digest the surrounding connective tissue and resulted in larger numbers of isolated islets (69;70). Attempts were made at developing an effective, reproducible protocol for use in human islet isolation but researchers struggled to produce consistent results, citing problems with batch to batch variability of collagenase preparations (71). In 1989, Warnock *et al* reported sustained insulin secretion for 10 weeks following an islet-kidney allotransplantation in humans, demonstrating that islet transplantation could become an

effective method of treatment in T1DM (72). Because of the success in solid organ transplantation, the combined triple immunosuppression regime using prednisone, azathioprine, and cyclosporine was applied in human islet transplantation. A major finding in the 1990's was that many immunosuppressants were toxic to isolated islets particularly the glucocorticoids (73;74). In 1999, an Edmonton based group successfully transplanted 7 diabetic patients with human islets using a glucocorticoid-free immunosuppressive protocol (Figure 1-2). The immunosuppressive treatment involved rapamycin, a low dose of tacrolimus, and a new drug, daclizumab (71;75;76). It was the first indication that islets could be successfully transplanted in a reproducible manner and correct diabetes in humans.

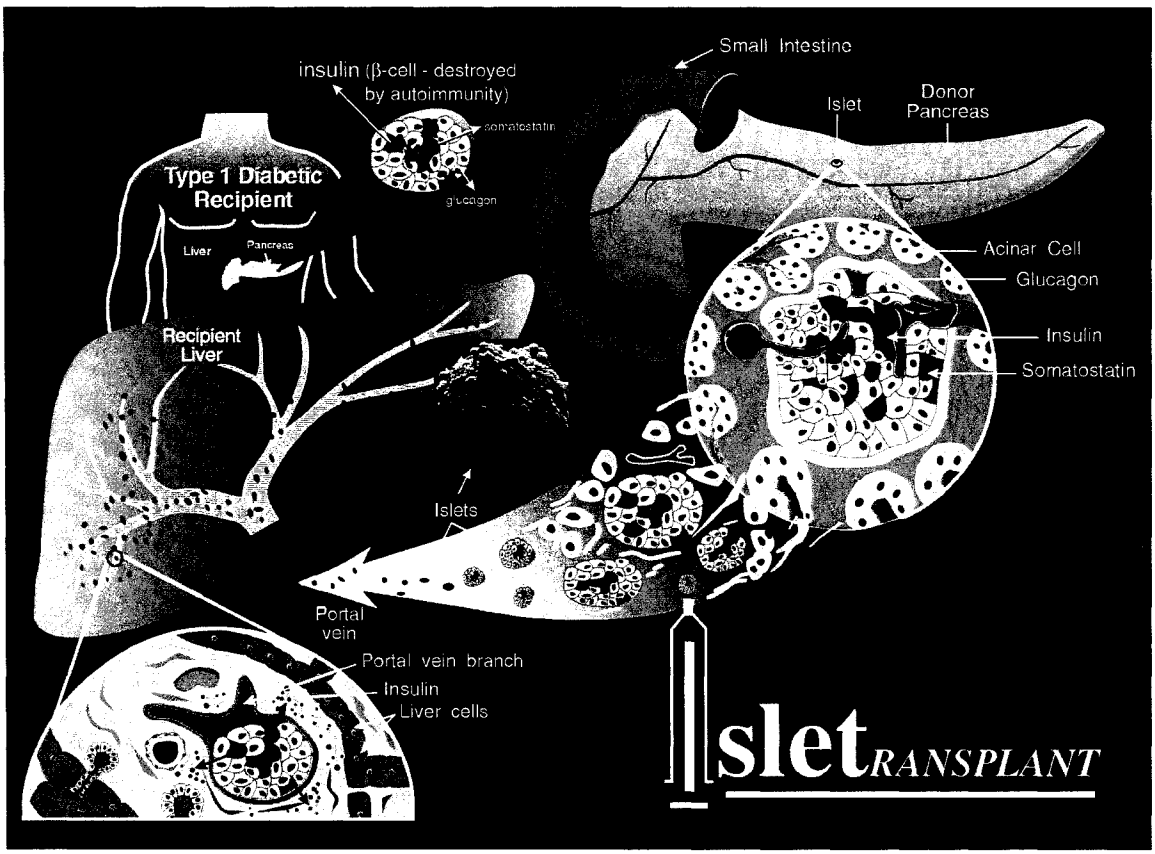


Figure 1-2. Human islet transplantation procedure.

The recent success in islet transplantation using the Edmonton protocol has provided support to researchers and patients about the viability of this approach in treating T1DM. At present, over 500 patients have received islet transplantations at over 50 institutions worldwide in the past 5 years (77). A five year follow-up study of patients that received an islet transplantation revealed improved glucose stability and near prevention of hypoglycemic episodes. Although the majority of patients after five years required exogenous insulin injections, C-peptide secretion was maintained after 5 years and hemoglobin A1c levels were markedly improved after 5 years when compared to A1c levels before the islet transplantation (75;78;79). Clinical islet transplantation is not without its drawbacks. Numerous side effects and complications are associated with the procedure and include but are not limited to: bleeding during transplantation, instant blood-mediated inflammatory reaction (IBMIR) events, and thrombosis (80-82). The need for continuous and chronic administration of immunosuppressive drugs limits the applicability of this procedure to a larger patient population. Immunosuppressive drugs are associated with a number of harmful side effects and can include increased risks of cancer and infection (83). Efforts are now being directed at discovering new ways of preventing the rejection of the transplanted islets that have fewer harmful effects to the patient and will be discussed further in section 1.5.

1.2.2 PANCREAS TRANSPLANTATION

Initial attempts at pancreas transplantation were met with difficulty largely due to the exocrine enzymes present in the pancreas which damaged the endocrine tissue and host tissue (84-86). The first human pancreas transplantation was performed in 1966

along with a simultaneous kidney transplant. Endocrine function was initially reported for several weeks, but eventually both organs were rejected (87).

Since then pancreas transplantation has blossomed and at present nearly 24,000 pancreas transplants have been performed worldwide as reported by the International Pancreas Transplant Registry. Pancreas transplantation in diabetes is typically done as a simultaneous kidney and pancreas transplant (SKP), although pancreas transplantation alone (PTA) and pancreas after kidney (PAK) transplantation are becoming more common. SKP transplantation at present however, yields the best graft survival rate with complete insulin independence rates after 1 year at 85%. The graft survival rate with complete insulin independence in PTA is less successful at only 60% at one year (88-91). SKP transplantation is often preferred especially if diabetes has been difficult to control (92). Pancreas transplantation in general, has been associated with an improvement in diabetic retinopathy, nephropathy, neuropathy and vasculopathy, but as with major surgeries, carries with it a significant risk of morbidity (91-95). Similar to islet transplantation, patients which undergo pancreas transplantation are required to continuously take immunosuppressive drugs, which are associated with a number of harmful side effects. This creates an ethical hurdle in patient's with T1DM, who are able to successfully manage their diabetes with exogenous insulin administration, making it difficult to justify pancreas transplantation given the risks of surgery and immunosuppression.

1.2.3 STEM CELL DIFFERENTIATION

The search for an unlimited supply of insulin producing tissue has lead researchers to the study of stem cells as a potential therapy for T1DM. It is believed that during embryonic development, a specific cell type, which is characterized by the expression of two transcription factors, Pdx-1 and neurogenin-3, gives rise to all islet cell types (96). The identification of a stem cell or precursor cell in the adult pancreas remains much more controversial. Recent evidence suggests that the pancreatic and hepatic cell types (hepatocytes, islet, acinar and ductal cells) have the ability to de- and trans-differentiate into each other under appropriate conditions (96). Several groups have reported successful differentiation of stem cells or precursor cells into insulin producing tissue (97-100). Recently, a group from China reported a procedure for differentiating embryonic stem cells into insulin producing cells using activin A, all-trans retinoic acid, and a milieu of other maturation factors. These insulin producing cells when transplanted into STZ induced diabetic mice were capable correcting diabetes and restoring normoglycemia (97). The emergence of human embryonic stem cells has led to an active area of research, and a potential source of insulin producing tissue, however developing reproducible methods for differentiating these precursor cells into insulin producing tissue are still being investigated (101-107).

1.2.4 β CELL REGENERATION

The endocrine portion of the pancreas recently, has been found to have the capacity to regenerate. This has prompted researchers to investigate methods of regenerating β cells with the goal of increasing β cell mass to restore euglycemia.

Increasing β cell mass can be accomplished through several ways including: β cell replication, increase in β cell size, decrease in β cell death, and β cell differentiation of existing β cell progenitors (103;104;108;109). A major question which limits the applicability of β cell regeneration, is the issue of whether auto-reactive T cells would target and destroy newly regenerated β cells (103). Indeed studies have shown that auto-reactive T cells persist in the body of the diabetic patient chronically, which has been demonstrated by experiments in which healthy islet cells transplanted into syngeneic, long-term diabetic mice or humans were quickly killed by these same auto-reactive T cells (110). Recently however, several studies have succeeded in regenerating β cells in the animal model for T1DM, the NOD mouse. One study found that transient treatment of overtly diabetic NOD mice with anti-lymphocyte serum to prevent autoimmunity, and exendin-4, resulted in complete remission of diabetes in 88% of mice within 75 days. This finding was accompanied by a progressive normalization of glucose tolerance, improved islet histology, increased insulin content in the pancreas, and insulin release in response to a glucose challenge (111). In another study, NOD mice that were treated with a combination of epidermal growth factor (EGF) and gastrin for 2 weeks, restored normoglycemia after diabetes onset in five of six mice (83%) for 10 weeks after the treatment was stopped. It was found that the combination of EGF and gastrin increases pancreatic beta-cell mass and reverses hyperglycemia in acutely diabetic NOD mice (112). The same combination of EGF and gastrin was also found to increase β cell mass in adult human pancreatic islets (113). A study published in 2003, discovered that the treatment of NOD mice with overt diabetes by injection of donor spleen cells and complete Freund's adjuvant, abrogates autoimmunity and permanently restores

normoglycemia. The authors reported that the return of endogenous insulin secretion was accompanied by the reappearance of pancreatic β cells. Interestingly, it was shown that spleen cells administered to diabetic NOD females contain cells which rapidly differentiate into islet and ductal epithelial cells within the pancreas (114). Clearly this is an area of rapid growth, however is has yet to be demonstrated that β cell regeneration is an effective and safe approach in non-human primates and in patients with T1DM.

1.3 CHALLENGES FOR ISLET TRANSPLANTATION IN T1DM

The success of the Edmonton Protocol has provided support for islet transplantation as a true alternative treatment for T1DM. Islet transplantation has proven to be remarkably successful in stabilizing glucose control to a degree that is greatly superior to even intensive insulin therapy (76).

At present however, islet transplantation is only a treatment alternative for a very select patient population, who experience extreme difficulty in controlling their blood glucose levels, often referred to as brittle diabetics. There are several barriers to the successful widespread implementation of islet transplantation. One is the shortage of donor pancreatic tissue. The second, is the immune mediated rejection of the transplant and the requirement for continuous immunosuppression. The third, is the potential of autoimmunity to act as a barrier to the successful engraftment of the islet transplant.

1.3.1 SHORTAGE OF DONOR TISSUE

As in almost all types of clinical transplantation, the number of potential recipients far outnumbers the number of donors, and in islet transplantation the situation

is no different. However, this shortage of donor pancreatic tissue is further compounded by the requirement of typically two or more donor pancreases to completely free patients from exogenous insulin administration (115-119). This requirement is due in part to the lack of engraftment of some of the transplanted islets. The lack of engraftment can be attributed to a number of factors including: hepatic steatosis, ischemic injury, and instant blood-mediated inflammatory reaction (IBMIR) (120-124). IBMIR is arguably the strongest barrier to islet engraftment. Human islets when exposed to blood elicit IBMIR which is characterized by platelet consumption, and activation of the coagulation and complement systems. The islets become surrounded by clots and infiltrated with leukocytes, and are damaged as evidenced by insulin dumping (124). Efforts are currently being directed at preventing IBMIR and at improving islet engraftment in order to alleviate some of the pressure on the shortage of cadaveric donors in clinical islet transplantation. Another potential solution to the shortage of donor pancreatic tissue is the use of animal sources of insulin producing tissue, referred to as xenotransplantation. Xenotransplantation will be discussed further in section 1.4.

1.3.2 IMMUNE MEDIATED REJECTION OF TRANSPLANTED ISLET GRAFTS

1.3.2.1 T Cell Mediated Immune Response

The T cell mediated immune response remains a formidable barrier to islet transplantation. In order for the T cell to become fully activated in response to donor antigen, the delivery of two separate but complimentary signals is required. Signal 1 is delivered during the cognate interaction between the T cell receptor (TCR)/CD3 complex and an MHC-bound peptide on an antigen presenting cell (APC) (125). The second signal

is an antigen nonspecific signal triggered by the interaction of a pair(s) of cell surface costimulatory molecules expressed on the T cell and APC which is termed Signal 2. It is important to note that costimulatory molecules cannot trigger T cell activation on their own. However, the interaction of costimulatory molecules is critical for proper T cell activation as TCR engagement in the absence of effective costimulation often results in T cell anergy and/or apoptosis (126-128).

In a transplantation setting, two pathways are capable of T cell activation. One, the direct pathway, wherein the host T cell recognizes antigen presented on the surface of donor APCs. In the second pathway, the indirect pathway, the T cell is activated by recognition of antigen that is presented by host APC (129). It has been well established that the indirect pathway of antigen presentation dominates when the phylogenetic disparity between the donor and recipient increase and that the direct pathway is increasingly active in the rejection of allogeneic and syngeneic transplants (130;131). These two pathways of antigen presentation are both capable of T cell activation and lead to an immune response, which results in the destruction of the transplanted islet graft. In order to prevent the activation of T cells following transplantation, presently immunosuppression is implemented, but because of the toxicity of immunosuppression, new ways of preventing the T cell mediated graft rejection are being investigated and will be discussed further in section 1.5.

1.3.2.2 Humoral Mediated Immune Response

Naturally occurring antibodies can be present in the serum of a transplant recipient which have specificity for antigens present on the transplanted graft. Typically this occurs from having prior exposure to the particular antigen or the antibody being

capable of recognizing a similar antigen. When pre-formed antibodies in the recipient recognize antigens present within the graft, this can lead to process referred to as hyperacute rejection. When the antibodies bind to their antigen on the transplanted graft, they induce graft rejection through Fc-mediated activation of the complement system and can result in graft rejection in as little as a few minutes (132). Humoral mediated rejection of transplants also occurs in an acute and chronic pathology. In acute and chronic rejection, antigens present on the graft are recognized by the corresponding B cell receptor. The B cell response requires the involvement of T_H cells which secrete cytokines that are critical for the proliferation and maturation of B cells into antibody secreting plasma cells. The binding of antibody to its target triggers the activation of complement, which subsequently destroys the antibody bound cell (133). Strategies to prevent humoral mediated rejection typically target T cells as T_H cells are typically required for the activation of B cells. Newer approaches are also under development which target co-stimulatory molecules present on B cells (134).

1.3.3 AUTOIMMUNITY

A major question which limits the applicability of islet transplantation, is the issue of whether auto-reactive T cells are capable of recognizing and destroying a transplanted islet graft (103;135-137). Studies have shown that auto-reactive T cells persist in the body of the diabetic patient indefinitely (110). In terms of graft rejection, it is often difficult to distinguish between immune mediated rejection or autoimmune mediated rejection. The NOD mouse has been an invaluable animal model of T1DM, however it is well known that the NOD mouse has several immune abnormalities (138-140), and some

have suggested that it may be resistant to tolerance induction (141). In 2001, it was confirmed that recurrent autoimmunity does contribute to islet allograft rejection and accelerates graft rejection in NOD mice (137). Despite this finding, success has been achieved in preventing the autoimmune destruction of transplanted islet grafts in the NOD mouse. NOD mice that were transplanted with allo- and syngeneic islets and treated with anti-lymphocyte serum (ALS) followed by co-administration of donor pancreatic lymph node cells (PLNCs) became normoglycemic and tolerated minor antigen-disparate islet grafts for >100 days and syngeneic islet grafts indefinitely (142). Another group found that a short course of anti-CD4 monoclonal antibody delayed the rejection of allogeneic islet grafts in diabetic female NOD mice. However, this therapy was unsuccessful at protecting the islet grafts long term (143).

The participation of autoimmunity in xenogeneic islet graft rejection is controversial. Some argue that xenogeneic islets could be targeted equally as efficiently by auto-reactive T cells, as allo or auto-islets are by a person with T1DM (144). A study published in 2003 however, reported that pig islet xenografts following a short course of anti-CD4 monoclonal antibody therapy, were resistant to autoimmune destruction by NOD mice (145). Unfortunately, no autoimmune model of diabetes exists in large animals, consequently, we can only extrapolate what role autoimmunity in patients with T1DM will have in the context of xenogeneic islet transplantation in the clinical setting.

1.4 XENOGENEIC TISSUE AS A SOURCE OF ISLETS

While many xenogeneic sources of insulin producing tissue have been used experimentally, pigs are arguably the most ideal source for humans. Pigs represent an

attractive source of islets for at least three reasons: First, pig and human islets are similar morphologically and pig insulin is structurally similar to human insulin (146). Second, the supply of pigs is potentially unlimited because of shortened gestation and large litters (147). Third, genetically engineered pigs lacking antigens readily recognized by the human immune system could prevent the extent of immunosuppression required to prevent rejection (148;149).

One of the major concerns in xenotransplantation is the risk of zoonotic diseases, specifically, the transmission of animal pathogens, particularly viruses, to recipients and the possible adaptation of such pathogens for human-to-human transmission (150). Porcine endogenous retrovirus (PERV) is a retrovirus which is endogenously expressed in pigs (151). PERV's are especially concerning because of their ability to infect human cells and because, presently, attempts to remove the virus from the porcine genome have been unsuccessful (152). Recently however, a study involving porcine islet transplantation in non-human primates, was unable to detect PERV dissemination in the recipient (153). Despite this encouraging finding, further research is required before safety assurances can be given in xenotransplantation (154-156).

Several groups have investigated the use of porcine islets to experimentally correct diabetes in a variety of animal models (147;157-161). However, what is not clear is whether it is more beneficial to transplant adult, newborn or fetal tissue.

1.4.1 ADULT PORCINE ISLETS

Proponents of the use of adult porcine tissue argue that a larger number of islets can be obtained from adult pancreases. In addition, the islets are mature at the time of

isolation, are larger in size, and the potential for engraftment is better (162). Adult porcine islets however, are less robust in tissue culture and more susceptible to ischemic damage (129;163). Adult porcine islets have been shown to reverse diabetes in a variety of animal models (163-169). Recently, adult porcine islets were shown to correct diabetes in non-human primates for more than 100 days after intraportal transplantation. The Cynomolgus macaques were treated with a cocktail of anti-rejection drugs in order to prevent rejection. The authors indicated that for even longer term success of the islet grafts, more selective targeting of indirect immune recognition and effector pathways is critical (170).

1.4.2 NEONATAL PORCINE ISLETS

Others believe that neonatal tissue constitutes the most ideal source of islets for human transplantation, which is based on five main reasons. First, the neonatal pancreas is less fibrous and easier to isolate, and consequently better islet yields can be obtained per gram of pancreatic tissue (147). Second, neonatal islets maintain considerable growth capacity, and may increase in number after subsequent transplantation (160). Third, the porcine islet preparation is not 100% pure. Ductal cells constitute a significant proportion of the isolated islet suspension, and these cells are hypothesized to be precursor cells of islet cells, giving rise to the potential for differentiation to insulin producing cells post-transplantation (147;171;172). Fourth, neonatal pigs would require fewer facilities and resources to house and maintain prior to transplantation than adult pigs, which would require months to mature. Lastly, the potential for maintaining adult pigs pathogen free would be less likely than that for newborn pigs given the increased

time required to mature a pig. Some disadvantages associated with neonatal pancreatic tissue include the inability of immature islets to immediately reverse hyperglycemia when transplanted as compared with mature islets. Another disadvantage with neonatal porcine islets is the presence of potential antigens on the surface of the islets, which could elicit an immune response by the recipient (129;173-176). Humans do not express Galα(1,3)Gal and consequently produce naturally occurring antibodies to Galα(1,3)Gal epitopes as a result of hypothesized sensitization by micro organisms which also co-express Galα(1,3)Gal epitopes (177;178). Pigs which represent the most attractive source of donor tissue to date contain an intact α1,3-galactosyltransferase (α1,3GT) gene and consequently express Galα(1,3)Gal. For solid organ transplantation this represents a major barrier to the use of xenogeneic tissue, termed hyperacute rejection, which is characterized by the binding of circulating anti-Galα(1,3)Gal antibodies, rapid activation of complement and coagulation cascades and the destruction of the vasculature of the transplanted organ. Galα(1,3)Gal is primarily expressed on the endothelium of the vasculature of porcine tissues. The expression of Galα(1,3)Gal on porcine islets remains controversial, however a recent study suggests that the expression of Galα(1,3)Gal on these islets is time dependent. It was found that the expression of Galα(1,3)Gal was higher in less mature neonatal porcine islets and that this expression decreased over time as the islets matured, and that by 200 days after transplantation, Galα(1,3)Gal expression was undetectable (173). While the expression of Galα(1,3)Gal on porcine tissue represents a significant concern, a recent study involving the transplantation of neonatal porcine islets into non-human primates, suggests that Galα(1,3)Gal mediated rejection may not prevent the use of porcine tissue in clinical islet transplantation. In this study, no

attempt to remove or block preformed Galα(1,3)Gal antibodies was made and it was found that no hyperacute rejection was observed and that of those recipients that eventually rejected the transplant, that this rejection was not associated with an increase in Galα(1,3)Gal antibody titres (153). Numerous studies have shown that neonatal porcine islets are capable of restoring normoglycemia in small and large animal models (129;157-159;176;179-182), however, this study was the first to demonstrate that neonatal porcine islets are able to restore normoglycemia in non-human primates. The authors found that Rhesus macaques that were transplanted and treated with a CD28-CD154 costimulation blockade regimen achieved sustained insulin independence for a median survival time of >140 days (153).

1.4.3 FETAL PORCINE ISLETS

Fetal porcine islets have many of the same attractive properties that neonatal porcine islets do including the ability of the immature islets to proliferate and resist ischemic damage while having poorly developed exocrine tissue. The lack of developed exocrine tissue is advantageous because the absence of developed exocrine cells can result in a delayed onset of autolysis following periods of ischemic damage and allow more flexibility in harvesting islets from the immature pancreas (174;183;184). However, like neonatal porcine islets, fetal porcine islets also express Galα(1,3)Gal, which could potentially mediate hyperacute rejection once transplanted (183;185;186). There is evidence to suggest that fetal porcine islets, like neonatal porcine islets may not undergo hyperacute rejection once transplanted. In 1998, a study was published in which human T1DM patients were transplanted with fetal porcine islets. A kidney biopsy

specimen obtained 3 weeks after transplantation still contained cells positive for insulin and others positive for glucagon and somatostatin (187;188). However, fetal islets in general have been found to exhibit a poor insulin secretory response to glucose, which is believed to be attributed to the excessive immaturity of the tissue (175;189-191).

1.4.4 XENOGENEIC ANIMAL MODELS IN ISLET TRANSPLANTATION

Porcine islets have been proposed as a suitable alternative source of islets in clinical transplantation (147;192-194). However, at present many experimental models involving porcine tissue utilize mice as recipients, which represents a discordant relationship (195). Discordancy, is defined as the potential for hyperacute rejection when donor tissue is transplanted into a recipient. Typically, a discordant relationship is one in which the donor is evolutionarily diverse when compared to the recipient (196;197). Pigs and humans are more closely related than pigs and mice (198), consequently the type of immune response which is elicited in each situation is different. This is especially evident as, anti-rejection therapies which are effective in mice receiving porcine islets are ineffective when applied to a different combination of donor and recipient. Graft rejection in concordant transplantation is strongly mediated by the cellular immune mechanisms. In discordant transplantation however, rejection is formidably mediated by humoral mechanisms (196). It has been well established that the indirect pathway of antigen presentation dominates in discordant transplantation and that the direct pathway is increasingly active in the rejection of allogeneic and syngeneic transplants (130;131). Therefore, examining the value of potential clinical therapies to prevent xenograft rejection and their ability to prevent concordant and discordant rejection is of significant

importance. If porcine islets are to become a source of islets in clinical transplantation, the immune response must be examined as the relation between donor and recipient becomes more concordant. Rat islets when transplanted into mice, provide an effective experimental model for studying concordant xenograft rejection. By elucidating the mechanisms of concordant xenograft rejection, this information can be applied to devising effective anti-rejection therapies for clinical islet transplantation.

1.5 STRATEGIES FOR PREVENTING THE IMMUNE REJECTION OF TRANSPLANTED ISLETS

1.5.1 IMMUNOSUPPRESSIVE DRUGS

Immunosuppressive agents have shaped the face of clinical transplantation as we know it. There are many classes of immunosuppressive drugs including: corticosteroids, calcineurin inhibitors, anti-metabolites and TOR inhibitors. The majority of immunosuppressive drugs act in the induction phase of an immune response and inhibit the activation and/or proliferation of lymphocytes. In the past, the calcineurin inhibitor, cyclosporine and corticosteroids formed the backbone of many immunosuppressive regimens. Cyclosporine is a fungal peptide which once absorbed, binds with the cytosolic protein, cyclophilin, to form a complex. This complex binds and inhibits calcineurin, a protein which is critical for the activation of transcription factors for the IL-2 gene. IL-2 has been found to be essential for the activation, proliferation and differentiation of T cells. Consequently, agents which prevent or interfere with IL-2 signaling are potent immunosuppressants (133;199;200). However, presently cyclosporine is rarely implemented in transplantation mainly due to its deleterious side

effects the most serious of which is nephrotoxicity (201). An important discovery in islet transplantation was the observation that cyclosporine and corticosteroids, in particular the glucocorticoids, are harmful to the islet transplantation and decrease the potential for successful engraftment (202-204). In clinical islet transplantation, effective immunosuppression has been instrumental to the success of the Edmonton Protocol. Newer immunosuppressive drugs have emerged with fewer detrimental side effects to the patient and the transplanted graft. At present, patients which undergo islet transplantation are placed on a novel immunosuppressive regimen which includes: rapamycin also referred to as sirolimus (discussed in section 1.6), low-dose tacrolimus, and an antibody specific to the IL-2 receptor (discussed in section 1.5.2) (205). Tacrolimus, a macrolide antibiotic, is another type of calcineurin inhibitor. Tacrolimus has a very similar mechanism of action as cyclosporine with two main differences. The first is that tacrolimus once absorbed, binds to FK-binding protein (FKBP) instead of cyclophilin, and this complex binds and inhibits calcineurin. The second is that tacrolimus is active at lower concentrations than cyclosporine, allowing physicians to administer the immunosuppressant with fewer associated side effects (200).

1.5.2 MONOCLONAL ANTIBODIES

T cell activation by T cell receptor recognition of antigen presented on MHC by an APC is not sufficient to fully activate naïve T cells. It is now commonly accepted that full T cell activation and the type of response requires additional interaction of co-stimulatory molecules present on the surfaces of T cells and APCs (206). Blocking of these additional signals by targeting co-stimulatory molecules prevents complete

activation of the T cell and represents an attractive therapeutic target of anti-rejection therapy. While some group monoclonal antibodies into immunosuppressive agents (201), myself and others prefer the term immunomodulatory given the specificity of their targets and their propensity for fewer side effects (207-212).

Targeting of T cells with monoclonal antibodies (mAb) has represented the focus of many anti-rejection regimens. The targeting of CD154 (CD40L) on T cells has been studied extensively as a means of preventing islet graft rejection. In allo-islet models, the blocking of CD154 along with PD-1 stimulation was successful at inducing long-term survival of MHC mismatched islet allografts (213). Targeting of CD154 in xenogeneic islet transplantation appears to be equally as efficacious. Treatment with cytotoxic T lymphocyte antigen-4 (CTLA-4) is effective in preventing the rejection of neonatal porcine islets transplanted into CD154 knockout mice (214), and additionally anti-CD154 monotherapy was effective at inducing tolerance to human islets transplanted into mice (215). Treatment with anti-CD154 and CTLA-4 was also effective at inducing indefinite rat islet xenograft survival in mice (216). Anti-CD154 monotherapy has been reported to be effective at inducing long-term survival of concordant and discordant islet xenografts in mice (217), although other groups have reported that anti-CD154 monotherapy does not prevent the rejection of neonatal porcine islets in the majority of transplanted mice (158).

Targeting of B7 co-stimulatory molecules with a soluble fusion protein, CTLA4Ig was effective at preventing human islet graft rejection when transplanted into mice (218). When CTLA4Ig therapy was combined with microencapsulation of neonatal porcine islets, graft function was prolonged and the sensitization of the host to the transplant was

delayed (219). Targeting of CD4 has also been shown to prolong both allo and xenogeneic islet transplantation and prevent the recurrence of autoimmune disease in NOD mice. In addition, non-depleting anti-CD4 was found to be superior to depleting anti-CD4 monoclonal antibody in preventing xenogeneic islet rejection (220).

Many of the monoclonal antibodies utilized in experimental models have not yet been applied clinically in islet transplantation, however monoclonal antibodies to the IL-2 receptor form part of the foundation of the anti-rejection regimen of the widely successful Edmonton Protocol (76). Anti-IL-2 receptor antibodies target the IL-2 receptor complex on activated T cells, and disrupt IL-2 signaling. This is in contrast to calcineurin inhibitors, which as discussed previously, exert their effect by inhibiting the calcineurin complex and preventing IL-2 gene activation (221). Combining antibodies to the IL-2 receptor with calcineurin inhibitors has resulted in greater graft survival in many types of transplantation (222-225), because of the ability of these anti-rejection therapies to act synergistically.

1.5.3 TOLERANCE INDUCTION

Tolerance is the specific immune unresponsiveness to an antigen or set of antigens which are normally immunogenic, while remaining immuno-sensitive to third party antigens, with the stipulation that there has been prior exposure to those specific antigens (226). There are two main approaches for the generation of tolerance, the generation of central tolerance and, the generation of peripheral tolerance. Typically approaches aimed at generating central tolerance employ methods of chimerism induction and/or thymic grafting of donor antigens. While the generation of central tolerance is

effective at negatively selecting against self reactive T cells and potentially, donor reactive T cells, it should be noted that a small but significant number of self reactive T cells escape negative selection only to be controlled by peripheral tolerance mechanisms, emphasizing the effectiveness of peripheral tolerance (227).

1.5.3.1 Mixed Chimerism

The induction of tolerance to donor xeno-antigens via the development of mixed chimerism represents an attractive method of preventing the rejection of xenogeneic islets (228;229). Many argue that the induction of chimerism is likely to be essential if xenotransplantation is to become widespread, given the vigorous immune response towards xenografts (230). The development of chimerism would potentially allow islet transplant recipients to stop taking any form of immunosuppression and may prevent pre-existing autoimmunity from destroying the transplanted islet graft (231). Mixed chimerism in allo-transplant models has been firmly demonstrated, however inducing chimerism in a xeno-transplant model has proven to be more challenging (232). Preliminary studies demonstrated that xenogeneic chimerism with a non-lethal approach was capable of inducing donor specific tolerance in a rat to mouse concordant transplantation model (233-236). Recently however, mixed chimerism which can induce donor-specific T-cell tolerance has been achieved in discordant xenotransplantation (230). One study reported that the generation of chimerism induced simultaneous tolerance among T cells and Gal-reactive B cells, which is of particular importance in porcine to human xenotransplantation (237). Other studies have found that treating recipients with either anti-CD3 monoclonal antibody or rapamycin facilitates bone marrow chimerism and tolerance induction (238;239). Unfortunately, the generation of

chimerism via bone marrow transplantation is not without its own drawbacks. Many protocols require the irradiation of the recipient, which would constitute a major hurdle to overcome ethically. Another significant concern is the potential for the development of graft versus host disease (GVHD) in which donor T cells present within the graft, attack the recipient resulting in multi-organ damage and morbidity(240-242). Clearly, further investigation is needed with safety assurances and demonstrated effectiveness in larger animal models and before this therapy can advance to the clinic.

1.5.3.2 Intrathymic Grafting of Donor Antigens

Intrathymic grafting typically involves the deliberate exposure of donor antigens in the thymus of the recipient in the hopes of re-educating the recipient's immune system to treat the donor tissue as self. The rationale behind this approach is based on the observation that the avidity/affinity of the T-cell receptor (TCR) and major histocompatibility complex (MHC) self-peptide interactions control positive and negative selection of T-cells in the thymus, such that thymocytes bearing a TCR that has a high affinity for self antigens presented by self MHC, results in the deletion of that thymocyte (243;244). It has been proposed therefore, that introducing donor antigens into the thymus while T cells are undergoing maturation, may induce central tolerance via deletion of donor reactive T cells. It has even been suggested that the thymus may represent an ideal site for the actual islet transplant (245). Intrathymic injection of allopeptides/allo-islets induces acquired tolerance in experimental animal models (246;247) and in some cases was successful in inducing permanent islet allograft survival (248;249). Progress using similar approaches in xenogeneic models has been less successful. Porcine thymic tissue has been successfully transplanted into baboons and

was capable of inducing xenogeneic hyporesponsiveness but was not successful at inducing tolerance (250). It has also been reported that xenogeneic swine thymic transplants can induce tolerance to swine antigens in mice, however this study demonstrated that T cells were tolerant of xenogeneic pig antigens *in vitro* only (251). Recently Yamamoto *et al.* reported a novel strategy for inducing xenogeneic tolerance via vascularized thymic lobe transplantation which was capable of promoting early thymopoiesis and donor-specific cellular unresponsiveness, however again this was demonstrated *in vitro* only (252). Another study reported that porcine thymic tissue transplanted into thymectomized mice mediated positive selection of T cells and that expression of porcine MHC was not critical for the maintenance of memory $CD4^+$ T cells found in the periphery (253).

1.5.3.3 Immunoregulatory Cells

There has been an enormous expansion of information and studies involving immunoregulatory cells in recent years (254). Many types of cells with a regulatory phenotype have been identified (255). It is clear from allo-islet transplantation models that regulatory cells play a critical role in tolerance. It has been well demonstrated that different protocols can induce tolerance to islet allografts when associated with the induction of $CD4^+CD25^+$ regulatory T cells (256-260). In xenogeneic models, $CD4^+CD25^+$ regulatory T cells have been shown to suppress the secretion of inflammatory cytokines and IL-2 as well as suppress T cell cytolytic responses against xenogeneic porcine cells *in vitro* (261). Another study found that regulatory T cells of a $CD8^+CD28^-$ phenotype reduced the capacity of xenoreactive T_H cells to secrete IL-2 and was capable of inducing anergy of these xenoreactive cells (262). Although a clear

mechanism as to how immunoregulatory cells function has yet to be determined, at present they represent an attractive area of exploitation in tolerance induction in xenogeneic islet transplantation.

1.5.4 IMMUNE ISOLATION DEVICES

Many devices exist with the function of isolating the transplanted tissue from the immune system of the host including: vascular perfusion devises, macroencapsulation, and microencapsulation (263;264).

Vascular perfusion devices are tubular structures normally with a wide bore in the center, large enough to allow the insertion of a blood vessel through the center. The transplant is then placed within a membrane which comprises the tubular structure surrounding the blood vessel. The association of the device with the vasculature ensures adequate oxygen and nutrient delivery to the transplanted tissue (265). There are several drawbacks to devices of this type including: induction of the coagulation cascade, poor glycemic control, as well as the requirement for major vascular surgery to implant the device (265;266).

Macroencapsulation involves the implantation of islets into a single device, which can be comprised of various biocompatible materials. These devices are designed to exclude larger immune cells which could potentially be harmful to the islets, while remaining permeable to insulin, glucose and other nutrients important for maintaining the viability of the islets (267). While macroencapsulation devices are capable of restoring euglycemia in diabetic experimental animals (268-273), there are several shortcomings

including: overgrowth resulting from poor biocompatibility, membrane rupture, poor glycemic control, and necrosis due to poor diffusion of nutrients to the islets (266;267).

Microencapsulation is perhaps the most favoured immunoisolation device in islet transplantation. In microencapsulation, individual islets are surrounded by a thin spherical, polymeric membrane, normally composed of agarose or alginate. The porosity of the membrane permits the entry of nutrients and oxygen and the diffusion of insulin out of the capsule. Furthermore, the semi-permeable nature of the membrane prevents high molecular weight molecules, such as immune cells and antibodies from penetrating the capsule and coming into contact with the encapsulated islets (274-276). It has been previously shown that islets placed in alginate microcapsules can be effective in preventing the destruction of the islets mediated by human antibody and complement *in vitro* (182). *In vivo*, several groups have shown that encapsulation can successfully protect allo- and xenogeneic islets from immune mediated and autoimmune mediated rejection (157;266;277-280). Despite these promising findings, islet microencapsulation is not without its drawbacks. Although microcapsules physically separate the islets from the recipients' immune cells, there is still the potential for islet antigens and chemokines to cross the alginate microcapsule membrane and trigger an immune response. Indeed, it has been previously reported that when microencapsulated islets are transplanted into immune competent recipients, that over time, the capsules become overgrown with immune cells suggesting that the immune system has been alerted to the presence of the graft (281). In addition, experiments involving microencapsulated islets have often been difficult to reproduce due to variations within the encapsulation process, purity and biocompatibility of the materials, islet viability, and capsule diameter (274;276;282-284).

1.5.5 CO-TRANSPLANTATION WITH SERTOLI CELLS

Another attractive method of preventing the immune rejection of transplanted islets involves co-transplanting Sertoli cells with islets to provide immunologic and trophic support to co-transplanted cells (285;286). Sertoli cells reside within the testes and form part of the seminiferous tubules. Their role is to supply beneficial factors and support the developing germ cells as well as prevent the germ cells from being eliminated by the host immune system. Sertoli cells are known to produce FasL, TGF-β, and clusterin (287-289), which are suspected to have immunoprotective, anti-inflammatory, and tolerizing properties (287;289-293). The engagement of FasL to its receptor, FasR, induces apoptosis in those cells expressing FasR (294;295). TGF-β has well demonstrated immunomodulatory/immunosuppressive effects on a wide range of cell types, and is hypothesized to play a critical role in the induction of tolerance (296-302). Although the mechanism by which clusterin protects cells remains unknown, studies have shown that clusterin is upregulated in response to cell death, suggesting that it may play a role in the protection of surviving cells after damage (289). Experimentally, Sertoli cells have been found to protect cotransplanted allogeneic or xenogeneic cells from immune mediated and autoimmune mediated rejection, and in some cases, results in prolonged islet graft survival (285;286;303-310). A possible drawback of co-transplanting Sertoli cells along with islets is the potential for the Sertoli cells to out-compete the islets for nutrients. It has been observed that Sertoli cells, transplanted along with islets, have the capacity to rapidly proliferate and crowd out neighboring islets, which could potentially leave the islets nutrient deprived and necrotic (personal observation).

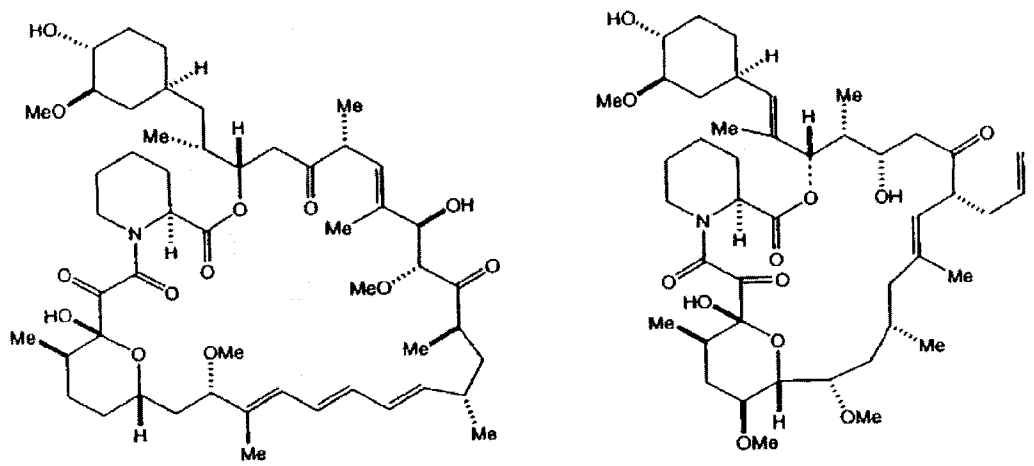
Another alternative to co-transplanting Sertoli cells with islets, is the use of the testis itself as the transplantation site (311;312). One study demonstrated prolonged islet xenograft survival when islets were transplanted into the testis of rats, however this therapy required supplementation with a short term course of cyclosporine A (311). Potential problems may arise if this approach continues towards the clinic. First, the testis may not represent an ideal transplantation site based on the large number of islets that are required to restore euglycemia and the relatively limited space which is available for the islets to engraft in the testis. Secondly, the potential for the activation of the immune system at the site of the testis may trigger collateral damage of the neighboring germ cells, potentially rendering the recipient sterile. Lastly, this approach is obviously only applicable to male patients, which would further limit the number of recipients who could potentially receive an islet transplant.

1.6 RAPAMYCIN (SIROLIMUS)

1.6.1 BACKGROUND

Rapamycin was first identified over thirty years ago during antibiotic screening tests conducted at Ayerst Research Laboratories. It is an anti-fungal metabolite produced by the bacteria *Streptomyces hygroscopicus* (313;314). Rapamycin derives its name from the native word for Easter Island, Rapa Nui, the place where *Streptomyces hygroscopicus* was first isolated in a soil sample (313;315). Although lacking antibacterial activity, rapamycin is a potent inhibitor of yeast growth and a moderate growth inhibitor of filamentous fungi (316). Its immunosuppressive properties were first identified from studies showing its inhibitory effects upon the production of humoral IgE as well as its

preventative effects in two animal models of human autoimmune disease, autoimmune encephalitis and adjuvant arthritis (317). Despite these observations, rapamycin did not gain widespread implementation as an immunosuppressive drug until it was discovered nearly 10 years later, that its structure was similar to another newly identified immunosuppressive drug, tacrolimus (FK506) (Figure 1-3). Interest in tacrolimus peaked when it was discovered that it possessed 100 times the potency of cyclosporine A (313). These two observations prompted researchers to reexamine the immunosuppressive potential of rapamycin.



Rapamycin Tacrolimus

Figure 1-3. Structural comparison of rapamycin and tacrolimus.

1.6.2 PHARMACOLOGY

Rapamycin is composed of a 31 membered lipophilic ring. The target of rapamycin has been identified and incidentally, in mammals is referred to as the mammalian target of rapamycin (mTOR) (318). Rapamycin does not directly bind

mTOR but elicits its effect by associating with FK-binding protein 12 (FKBP12), this complex then goes on to bind and inhibit mTOR (313;316;318). mTOR is a serine/threonine kinase, which regulates cell growth in response to nutrients, and has been reported to be a crucial regulator of protein synthesis and translation initiation (313) (Figure 1-4). TOR homologs have also been discovered in *Drosophila*, *Caenorhabditis elegans*, fungus, and plants (319-323), making TOR an evolutionarily conserved protein. The two main targets of mTOR are p70 ribosomal S6 kinase (SK61) and 4E-binding protein-1 (4E-BP1). The mTOR kinase in response to growth signals such as amino acids or growth factors, phosphorylates these substrates, inducing the activation of SK61 and inhibition of 4E-BP1. The function of the SK61 protein is the phosphorylation of the 40S ribosomal protein and subsequent activation of translation. 4E-BP1 however, acts as a translation inhibitor. When it is phosphorylated 4E-BP1 becomes inactivated. When mTOR phosphorylates 4E-BP1, it induces its dissociation from eukaryotic translation initiation factor 4E (eIF4E). Consequently, eIF4E is freed and can bind the cap structure at the 5' termini of mRNA's thereby allowing cap-dependent translation (324-327). Rapamycin by blocking mTOR, prevents protein synthesis, arresting the cell in the G1 phase of the cell cycle. It is a particularly potent inhibitor of T and B cell proliferation especially that which is induced by the interleukins (313).

Rapamycin is typically administered orally. After administration, rapamycin reaches its peak concentration in approximately 1 hour. The majority of rapamycin is bound to formed blood elements, in particular to human plasma proteins (~92%) the majority of those plasma proteins being serum albumin (97%) (328;329). The half life of the drug in renal transplant patients is 62 hrs. Rapamycin is metabolized by the same

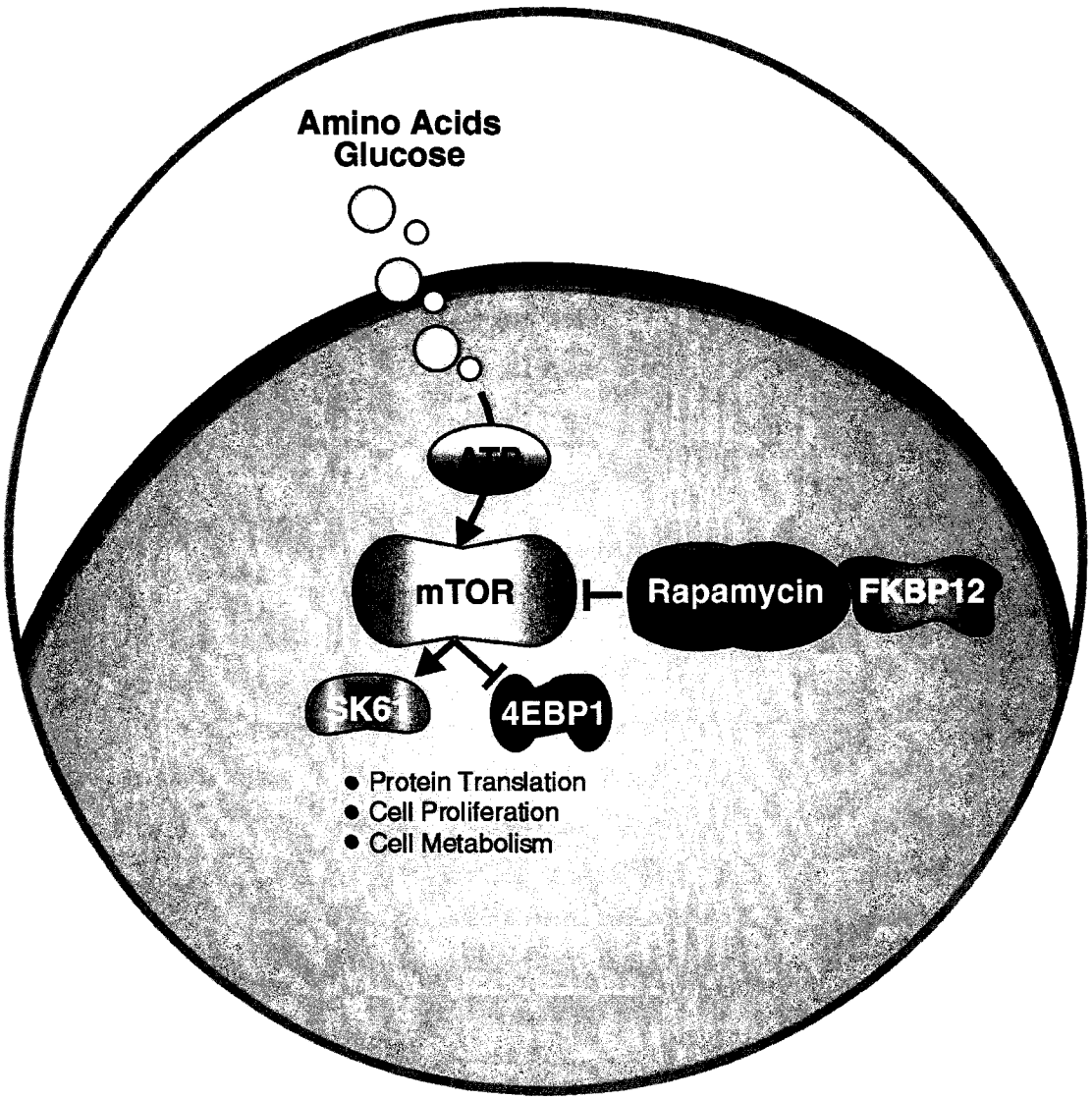


Figure 1-4. Mechanism of action of rapamycin.

cytochrome P450 3A enzyme involved in the metabolism of cyclosporine A and tacrolimus (FK506). The metabolism of rapamycin is primarily via simple demethylations and hydroxylations and is metabolized into 7 major metabolites (328). While some of these metabolites have mild efficacy, over 90% of the immunosuppressant

activity is due to rapamycin. The majority of rapamycin is secreted in the feces (91%) with ~ 2% being excreted in the urine (328;329).

Rapamycin administration like other immunosuppressive drugs is associated with potentially harmful side effects which can include: leukcopenia, thrombocytopenia, hypertriglyceridemia, hypercholesterolemia, mouth ulceration, ulceration of the small bowel, peripheral edema, joint pain, pulmonary fibrosis, wound infections and wound dehiscence (81;82;330-334).

1.6.3 LITERATURE

Rapamycin currently forms part of the immunosuppressive regimen administered to patients undergoing islet transplantation in the Edmonton Protocol. Currently, patients receiving an islet transplantation are treated with a loading dose of 0.2mg/kg followed by 0.1 mg/kg daily (76;335). While the discovery of rapamycin has been pivotal to the success of the Edmonton Protocol, rapamycin has also left its mark in other areas of medicine as well. Rapamcyin prescription has increased in kidney, pancreas, heart and liver transplantation in recent years (201). In addition, rapamycin has also been used to coat cardiac stents to prevent re-stenosis (336). Recently, rapamycin has gained attention from cancer researchers because of its ability to arrest cells in G1 of the cell cycle. It has been proposed that rapamycin may be an effective anti-cancer treatment in various types of cancers including: leukemia, breast and lung cancers (337-341).

Immunosuppressants have largely been thought of as a barrier to tolerance induction (227). New findings suggest that this may not be the case for rapamycin. A study published recently found that rapamycin treatment significantly extends the

window of opportunity for veto-based induction of tolerance to transplantation antigens (342). Rapamycin in conjunction with IL-10 therapy has been found to prevent allograft rejection as well as induce antigen-specific tolerance which can be transferred via adoptive transfer of $CD4^+$ cells (343). In addition, rapamycin combined with IL-10 is reported to efficiently block type 1 diabetes development and induce long-term immune tolerance in the absence of chronic immunosuppression in NOD mice. It was found following treatment with rapamycin, that an increase in the accumulation of $CD4^+CD25^+$Foxp3 regulatory T cells was detected in the pancreas of the NOD mice and this was attributed for the prevention of diabetes in these mice (344). Another interesting study which was published recently, reported that rapamycin selectively expands murine naturally occurring $CD4^+CD25^+$Foxp3 regulatory T cells in vitro, alluding to a potential ex vivo cellular therapy which could be applied in T1DM (345).

1.7 LEUKOCYTE FUNCTION ASSOCIATED ANTIGEN–1

1.7.1 EXPRESSION AND FUNCTION

Leukocyte function associated antigen-1 (LFA-1) (CD11a/CD18) is a member of a family of structurally and functionally similar leukocyte differentiation antigens and was first identified in mice in 1981 (346). This is a family of high molecular weight heterodimeric glycoproteins which share a β subunit of 95 kDa. This subunit is noncovalently bound to an α chain of which there are many different subtypes ranging in size from 150-175 kDa (347;348). The proteins are highly conserved in many species and their importance in cell to cell interactions and immune responses is demonstrated by the high incidence of infection in patients with congenital defects in these proteins (349).

LFA-1 is widely expressed on hematopoietic cells including: T and B lymphocytes, natural killer cells, monocytes, macrophages and granulocytes, but is most highly expressed on T cells followed by B cells (347-351). The initial interaction of a T cell receptor with its MHC presented antigen is not strong enough on its own to allow activation of the T cell (133). LFA-1 present on T cells acts synergistically to strengthen the binding of the T cell to the APC and facilitates the signalling which leads to the activation of the T cell. LFA-1 is the ligand of intercellular adhesion molecule-1 (ICAM-1) which is typically found on APCs and target cells (352). In the presence of antibody to LFA-1, ICAM-1 and LFA-1 cannot bind and the interaction between the T cell receptor and the antigen-MHC complex is inhibited or weakened. Consequently, the T cell cannot become fully activated and may become anergic or apoptotic (210). LFA-1 through a similar mechanism has also be shown to lower the threshold of B cell activation by facilitating B cell adhesion and synapse formation (353).

In addition to the previously specified role, LFA-1 also provides signals that promote T cell activation and differentiation. LFA-1 engagement contributes to CD3 and CD28 costimulation through a distinct signalling pathway. Upon engagement of LFA-1 and ICAM-1, the β2 chain of LFA-1 becomes phosphorylated, which ultimately leads to the promotion of c-Jun phosphorylation and the activation of IL-2 production (352;354;355). It has also been reported that LFA-1 engagement through this signalling pathway can polarize the T cell towards a T_H1 phenotype (354). In the presence of antibodies to LFA-1, the previously mentioned pathway is blocked, as a result, the LFA-1 mediated costimulation is prevented, potentially preventing the activation of the T cell (356).

The primary role of LFA-1 is as an integrin, and as such, is involved in the adhesion and migration of lymphocytes to endothelial cells in response to the activation of the immune system (357) (Figure 1-5). Activated T cells migrate from the blood into nonlymphoid tissues through a multistep process that involves cell rolling, arrest, and transmigration. Rolling cells are arrested through a firm adhesion step mediated in part by LFA-1. Once the cell has arrested against the endothelial wall, the cell begins to migrate through the endothelial wall and toward the site of immune activation (358-364). Therefore, antibodies to LFA-1 are also effective at preventing graft rejection, because they can disrupt lymphocyte homing to site of a transplanted graft (362).

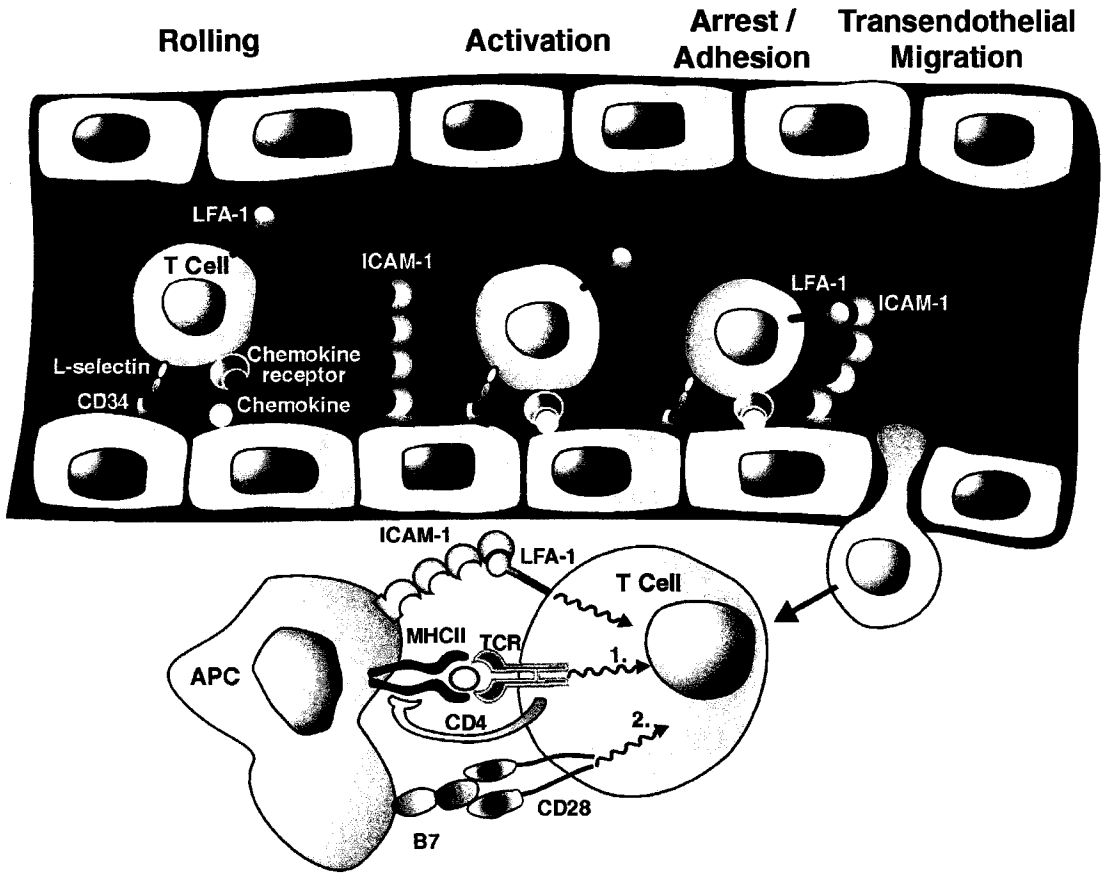


Figure 1-5. The role of LFA-1 in the immune response. LFA-1 facilitates the migration of leukocytes to the site of an immune response, stabilizes the APC/T cell immune synapse, and produces its own positive activation signal.

1.7.2 TARGETING OF LFA-1 AS A THERAPEUTIC STRATEGY

Numerous studies have demonstrated that antibodies directed towards LFA-1 inhibit T cell mediated killing, antigen-nonspecific natural killing and numerous other processes which are dependent upon cell to cell interaction for cellular function and ultimately improve islet graft survival (158;210;350;365-369). It has been reported that treatment with anti-LFA-1 and anti-ICAM-1 prevents the onset of diabetes in mice. This finding emphasizes the importance of the interaction of LFA-1 and ICAM-1 in T cell

activation and the initiation of the immune response in autoimmune diabetes (350;368). Antibodies to LFA-1 are normally combined with other costimulation blockade therapies in order to increase the potency of the anti-rejection regimen. Anti-LFA-1 combined with anti-CD154 has been shown to protect neonatal porcine islet xenografts (158), and induce dominant transplantation tolerance to islet allografts in a mechanism which is independent of IFN-γ or IL-4 secretion, the two prototypic Th1 and Th2 cytokines (366;369). Recently a humanized IgG1 form of the anti-LFA-1 antibody, Efalizumab, has become available (370). Efalizumab has shown marked efficacy in the treatment of psoriasis and is currently in phase IV of clinical trials. Efalizumab treatment is associated with some side effects, which can include: headache, chills, fever, nausea, vomiting and myalgia. Following clinical reports, the FDA and Genentech Inc revised the safety warnings for this drug to include immune-mediated hemolytic anemia (370). While Eflalizumab is not the primary indicated therapy for psoriasis, it does provide an attractive alternative for those patients who are intolerant of currently indicated treatments.

1.8 OBJECTIVES AND GENERAL OUTLINE

The main objective of this thesis was to examine the efficacy of rapamycin and anti-LFA-1 in the prevention of rat islet xenograft rejection in mice and the potential for the induction of tolerance. Additionally, the mechanism by which rapamycin and anti-LFA-1 therapy protects against xenograft rejection was also investigated.

A previous pilot study by our lab demonstrated that rapamycin and anti-LFA-1 provides protection from graft rejection when rat islets are transplanted into BALB/c

mice. The focus of Chapter 2 was to re-examine this combination of anti-rejection therapies in the same xenogeneic model and elucidate the mechanism by which this combination prevents the rejection of the xenogeneic islets.

In Chapter 3, the potential of rapamycin and anti-LFA-1 therapy to induce tolerance to rat islet xenografts was examined. Two different approaches were examined to answer this question. The first utilized spleen cells isolated from mice receiving rapamycin and anti-LFA-1, and were adoptively transferred to BALB/c rag-/- mice which were transplanted with the same rat islet xenograft. The second approach, involved performing a nephrectomy of the islet graft bearing kidney of mice that received rapamycin and anti-LFA-1 and re-challenging the same animal with an identical islet transplant in the contralateral kidney.

Islet transplantation is a treatment alternative to T1DM which is in its infancy. Unfortunately at present, the number of patients who could benefit from this therapy significantly outnumbers the supply of islets. In addition, the harsh immunosuppression required to prevent the rejection of the transplanted islet graft creates an ethical barrier which prevents islet transplantation from being applied to patients who can successfully manage their diabetes with exogenous insulin administration. Xenotransplantation has the potential to alleviate the supply shortage of donor islets, and with the advent of new and more specific immunomodulatory and immunosuppressive agents the goal of widespread islet transplantation is more a reality than ever.

1.9 REFERENCES

1. Pickup JC and Williams G. 2003. Textbook of Diabetes. Blackwell, Malden.

2. Daneman,D. 2006. Type 1 diabetes. *Lancet* 367:847-858.

3. Haller,M.J., Atkinson,M.A., and Schatz,D. 2005. Type 1 diabetes mellitus: etiology, presentation, and management. *Pediatr.Clin.North Am.* 52:1553-1578.

4. Narendran,P., Estella,E., and Fourlanos,S. 2005. Immunology of type 1 diabetes. *QJM.* 98:547-556.

5. King,K.M. 2003. A history of insulin: from discovery to modern alternatives. *Br.J.Nurs.* 12:1137-1141.

6. King,K.M. and Rubin,G. 2003. A history of diabetes: from antiquity to discovering insulin. *Br.J.Nurs.* 12:1091-1095.

7. Turina,M., Christ-Crain,M., and Polk,H.C., Jr. 2005. Impact of diabetes mellitus and metabolic disorders. *Surg.Clin.North Am.* 85:1153-61, ix.

8. Tripathi,B.K. and Srivastava,A.K. 2006. Diabetes mellitus: Complications and therapeutics. *Med.Sci.Monit.* 12:RA130-RA147.

9. Russell,T.A. 2006. Diabetic nephropathy in patients with type 1 diabetes mellitus. *Nephrol.Nurs.J.* 33:15-28.

10. Bober,E. and Buyukgebiz,A. 2005. Hypoglycemia and its effects on the brain in children with type 1 diabetes mellitus. *Pediatr.Endocrinol.Rev.* 2:378-382.

11. Perkins,B.A. and Krolewski,A.S. 2005. Early nephropathy in type 1 diabetes: a new perspective on who will and who will not progress. *Curr.Diab.Rep.* 5:455-463.

12. Marre,M. 2002. Nephropathy in type 1 diabetes. *Semin.Vasc.Med.* 2:97-107.

13. Shore,A.C. 2002. The microvasculature in type 1 diabetes. *Semin.Vasc.Med.* 2:9-20.

14. Rossing,P. 2005. The changing epidemiology of diabetic microangiopathy in type 1 diabetes. *Diabetologia* 48:1439-1444.

15. Libby,P., Nathan,D.M., Abraham,K., Brunzell,J.D., Fradkin,J.E., Haffner,S.M., Hsueh,W., Rewers,M., Roberts,B.T., Savage,P.J. *et al.* 2005. Report of the National Heart, Lung, and Blood Institute-National Institute of Diabetes and

Digestive and Kidney Diseases Working Group on Cardiovascular Complications of Type 1 Diabetes Mellitus. *Circulation* 111:3489-3493.

16. Dahl-Jorgensen,K., Larsen,J.R., and Hanssen,K.F. 2005. Atherosclerosis in childhood and adolescent type 1 diabetes: early disease, early treatment? *Diabetologia* 48:1445-1453.

17. Yoon,J.W. and Jun,H.S. 2001. Cellular and molecular pathogenic mechanisms of insulin-dependent diabetes mellitus. *Ann.N.Y.Acad.Sci.* 928:200-211.

18. Durinovic-Bello,I. 1998. Autoimmune diabetes: the role of T cells, MHC molecules and autoantigens. *Autoimmunity* 27:159-177.

19. Barker,J.M. 2006. Clinical review: Type 1 diabetes-associated autoimmunity: natural history, genetic associations, and screening. *J.Clin.Endocrinol.Metab* 91:1210-1217.

20. Kim,M.S. and Polychronakos,C. 2005. Immunogenetics of type 1 diabetes. *Horm.Res.* 64:180-188.

21. She,J.X. 1996. Susceptibility to type I diabetes: HLA-DQ and DR revisited. *Immunol.Today* 17:323-329.

22. Nepom,G.T. and Kwok,W.W. 1998. Molecular basis for HLA-DQ associations with IDDM. *Diabetes* 47:1177-1184.

23. Maier,L.M. and Wicker,L.S. 2005. Genetic susceptibility to type 1 diabetes. *Curr.Opin.Immunol.* 17:601-608.

24. Undlien,D.E., Friede,T., Rammensee,H.G., Joner,G., Dahl-Jorgensen,K., Sovik,O., Akselsen,H.E., Knutsen,I., Ronningen,K.S., and Thorsby,E. 1997. HLA-encoded genetic predisposition in IDDM: DR4 subtypes may be associated with different degrees of protection. *Diabetes* 46:143-149.

25. Schipper,R.F., Koeleman,B.P., Bruining,G.J., Schreuder,G.M., Verduijn,W., de Vries,R.R., and Roep,B.O. 2001. HLA class II associations with Type 1 diabetes mellitus: a multivariate approach. *Tissue Antigens* 57:144-150.

26. Roep,B.O., Schipper,R., Verduyn,W., Bruining,G.J., Schreuder,G.M., and de Vries,R.R. 1999. HLA-DRB1*0403 is associated with dominant protection against IDDM in the general Dutch population and subjects with high-risk DQA1*0301-DQB1*0302/DQA1*0501-DQB1*0201 genotype. *Tissue Antigens* 54:88-90.

27. Van der,A.B., Van Waeyenberge,C., Schuit,F., Heimberg,H., Vandewalle,C., Gorus,F., and Flament,J. 1995. DRB1*0403 protects against IDDM in Caucasians with the high-risk heterozygous DQA1*0301-DQB1*0302/DQA1*0501-DQB1*0201 genotype. Belgian Diabetes Registry. *Diabetes* 44:527-530.

28. Filippi,C. and von Herrath,M. 2005. How viral infections affect the autoimmune process leading to type 1 diabetes. *Cell Immunol.* 233:125-132.

29. Ginsberg-Fellner,F., Witt,M.E., Yagihashi,S., Dobersen,M.J., Taub,F., Fedun,B., McEvoy,R.C., Roman,S.H., Davies,R.G., Cooper,L.Z. *et al.* 1984. Congenital rubella syndrome as a model for type 1 (insulin-dependent) diabetes mellitus: increased prevalence of islet cell surface antibodies. *Diabetologia* 27 Suppl:87-89.

30. Karounos,D.G., Wolinsky,J.S., and Thomas,J.W. 1993. Monoclonal antibody to rubella virus capsid protein recognizes a beta-cell antigen. *J.Immunol.* 150:3080-3085.

31. Kaufman,D.L., Erlander,M.G., Clare-Salzler,M., Atkinson,M.A., Maclaren,N.K., and Tobin,A.J. 1992. Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus. *J.Clin.Invest* 89:283-292.

32. Cavallo,M.G., Baroni,M.G., Toto,A., Gearing,A.J., Forsey,T., Andreani,D., Thorpe,R., and Pozzilli,P. 1992. Viral infection induces cytokine release by beta islet cells. *Immunology* 75:664-668.

33. Hiemstra,H.S., Schloot,N.C., van Veelen,P.A., Willemen,S.J., Franken,K.L., van Rood,J.J., de Vries,R.R., Chaudhuri,A., Behan,P.O., Drijfhout,J.W. *et al.* 2001. Cytomegalovirus in autoimmunity: T cell crossreactivity to viral antigen and autoantigen glutamic acid decarboxylase. *Proc.Natl.Acad.Sci.U.S.A* 98:3988-3991.

34. Vaarala,O. 2005. Is type 1 diabetes a disease of the gut immune system triggered by cow's milk insulin? *Adv.Exp.Med.Biol.* 569:151-156.

35. Borch-Johnsen,K., Joner,G., Mandrup-Poulsen,T., Christy,M., Zachau-Christiansen,B., Kastrup,K., and Nerup,J. 1984. Relation between breast-feeding and incidence rates of insulin-dependent diabetes mellitus. A hypothesis. *Lancet* 2:1083-1086.

36. Scott,F.W., Norris,J.M., and Kolb,H. 1996. Milk and type I diabetes. *Diabetes Care* 19:379-383.

37. Norris,J.M. and Scott,F.W. 1996. A meta-analysis of infant diet and insulin-dependent diabetes mellitus: do biases play a role? *Epidemiology* 7:87-92.

38. Paronen,J., Knip,M., Savilahti,E., Virtanen,S.M., Ilonen,J., Akerblom,H.K., and Vaarala,O. 2000. Effect of cow's milk exposure and maternal type 1 diabetes on cellular and humoral immunization to dietary insulin in infants at genetic risk for type 1 diabetes. Finnish Trial to Reduce IDDM in the Genetically at Risk Study Group. *Diabetes* 49:1657-1665.

39. Vaarala,O., Knip,M., Paronen,J., Hamalainen,A.M., Muona,P., Vaatainen,M., Ilonen,J., Simell,O., and Akerblom,H.K. 1999. Cow's milk formula feeding

induces primary immunization to insulin in infants at genetic risk for type 1 diabetes. *Diabetes* 48:1389-1394.

40. Vaarala,O., Paronen,J., Otonkoski,T., and Akerblom,H.K. 1998. Cow milk feeding induces antibodies to insulin in children--a link between cow milk and insulin-dependent diabetes mellitus? *Scand.J.Immunol.* 47:131-135.

41. Shehadeh,N., Gelertner,L., Blazer,S., Perlman,R., Solovachik,L., and Etzioni,A. 2001. Importance of insulin content in infant diet: suggestion for a new infant formula. *Acta Paediatr.* 90:93-95.

42. Maron,R., Guerau-de-Arellano,M., Zhang,X., and Weiner,H.L. 2001. Oral administration of insulin to neonates suppresses spontaneous and cyclophosphamide induced diabetes in the NOD mouse. *J.Autoimmun.* 16:21-28.

43. Helgason,T. and Jonasson,M.R. 1981. Evidence for a food additive as a cause of ketosis-prone diabetes. *Lancet* 2:716-720.

44. Kostraba,J.N., Gay,E.C., Rewers,M., and Hamman,R.F. 1992. Nitrate levels in community drinking waters and risk of IDDM. An ecological analysis. *Diabetes Care* 15:1505-1508.

45. Scott,F.W. 1996. Food-induced type 1 diabetes in the BB rat. *Diabetes Metab Rev.* 12:341-359.

46. Scott,F.W., Sarwar,G., and Cloutier,H.E. 1988. Diabetogenicity of various protein sources in the diet of the diabetes-prone BB rat. *Adv.Exp.Med.Biol.* 246:277-285.

47. Lampasona,V., Bonfanti,R., Bazzigaluppi,E., Venerando,A., Chiumello,G., Bosi,E., and Bonifacio,E. 1999. Antibodies to tissue transglutaminase C in type I diabetes. *Diabetologia* 42:1195-1198.

48. Bao,F., Yu,L., Babu,S., Wang,T., Hoffenberg,E.J., Rewers,M., and Eisenbarth,G.S. 1999. One third of HLA DQ2 homozygous patients with type 1 diabetes express celiac disease-associated transglutaminase autoantibodies. *J.Autoimmun.* 13:143-148.

49. Dieterich,W., Ehnis,T., Bauer,M., Donner,P., Volta,U., Riecken,E.O., and Schuppan,D. 1997. Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat.Med.* 3:797-801.

50. 2000. Principles of Physiology. Mosby, Toronto.

51. Van De Graaff KM. 2006. Human Anatomy. McGraw-Hill, New York.

52. Orci,L. 1976. The microanatomy of the islets of Langerhans. *Metabolism* 25:1303-1313.

53. Rahier,J., Wallon,J., and Henquin,J.C. 1981. Cell populations in the endocrine pancreas of human neonates and infants. *Diabetologia* 20:540-546.

54. 1992. Pancreatic Islet Cell Transplantation. RG Landes Company, Georgetown.

55. Chernick,S.S., Clark,C.M., Jr., Gardiner,R.J., and Scow,R.O. 1972. Role of lipolytic and glucocorticoid hormones in the development of diabetic ketosis. *Diabetes* 21:946-954.

56. Schade,D.S. and Eaton,R.P. 1979. Prevention of diabetic ketoacidosis. *JAMA* 242:2455-2458.

57. Schade,D.S. and Eaton,R.P. 1979. Pathogenesis of diabetic ketoacidosis: a reappraisal. *Diabetes Care* 2:296-306.

58. Felig,P., Wahren,J., Sherwin,R., and Palaiologos,G. 1977. Amino acid and protein metabolism in diabetes mellitus. *Arch.Intern.Med.* 137:507-513.

59. Nair,K.S., Garrow,J.S., Ford,C., Mahler,R.F., and Halliday,D. 1983. Effect of poor diabetic control and obesity on whole body protein metabolism in man. *Diabetologia* 25:400-403.

60. DeWitt,D.E. and Hirsch,I.B. 2003. Outpatient insulin therapy in type 1 and type 2 diabetes mellitus: scientific review. *JAMA* 289:2254-2264.

61. Von Mering J and Minkowski O. 1889. Diabetes mellitus after pancreas extirpation. *Archiv fur Experimentale Patholgie und Pharmacologie* 26:111-127.

62. Nason,R.W., Rajotte,R.V., and Warnock,G.L. 1988. Pancreatic islet cell transplantation: past, present and future. *Diabetes Res.* 7:1-11.

63. Hellerstrom C. 1964. A method for the microdissection of intact pancreatic islets of mammals. *Acta Endocrinol* 45:122-132.

64. MOSKALEWSKI,S. 1965. ISOLATION AND CULTURE OF THE ISLETS OF LANGERHANS OF THE GUINEA PIG. *Gen.Comp Endocrinol.* 44:342-353.

65. Lacy,P.E. and Kostianovsky,M. 1967. Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes* 16:35-39.

66. Scharp,D.W., Kemp,C.B., Knight,M.J., Ballinger,W.F., and Lacy,P.E. 1973. The use of ficoll in the preparation of viable islets of langerhans from the rat pancreas. *Transplantation* 16:686-689.

67. Younoszai R, Sorensen RL, and Lindall AW. 1970. Homotransplantation of isolated pancreatic islets. *Diabetes* Suppl1:406 (Abstr.)

68. Ballinger,W.F. and Lacy,P.E. 1972. Transplantation of intact pancreatic islets in rats. *Surgery* 72:175-186.

69. Scharp,D.W., Downing,R., Merrell,R.C., and Greider,M. 1980. Isolating the elusive islet. *Diabetes* 29 Suppl 1:19-30.

70. Gray,D.W., McShane,P., Grant,A., and Morris,P.J. 1984. A method for isolation of islets of Langerhans from the human pancreas. *Diabetes* 33:1055-1061.

71. Robertson,R.P. 2001. Pancreatic islet transplantation for diabetes: successes, limitations, and challenges for the future. *Mol.Genet.Metab* 74:200-205.

72. Warnock,G.L., Kneteman,N.M., Ryan,E.A., Evans,M.G., Seelis,R.E., Halloran,P.F., Rabinovitch,A., and Rajotte,R.V. 1989. Continued function of pancreatic islets after transplantation in type I diabetes. *Lancet* 2:570-572.

73. Ohneda,M., Johnson,J.H., Inman,L.R., and Unger,R.H. 1993. GLUT-2 function in glucose-unresponsive beta cells of dexamethasone-induced diabetes in rats. *J.Clin.Invest* 92:1950-1956.

74. Morel,P., Kaufman,D.B., Field,M.J., Lloveras,J.K., Matas,A.J., and Sutherland,D.E. 1992. Detrimental effect of prednisone on canine islet autograft function. *Transplant.Proc.* 24:1048-1050.

75. Ryan,E.A., Lakey,J.R., Rajotte,R.V., Korbutt,G.S., Kin,T., Imes,S., Rabinovitch,A., Elliott,J.F., Bigam,D., Kneteman,N.M. *et al.* 2001. Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes* 50:710-719.

76. Shapiro,A.M., Lakey,J.R., Ryan,E.A., Korbutt,G.S., Toth,E., Warnock,G.L., Kneteman,N.M., and Rajotte,R.V. 2000. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N.Engl.J.Med.* 343:230-238.

77. Truong,W. and Shapiro,A.M. 2006. Progress in islet transplantation in patients with type 1 diabetes mellitus. *Treat.Endocrinol.* 5:147-158.

78. Ryan,E.A., Lakey,J.R., Paty,B.W., Imes,S., Korbutt,G.S., Kneteman,N.M., Bigam,D., Rajotte,R.V., and Shapiro,A.M. 2002. Successful islet transplantation: continued insulin reserve provides long-term glycemic control. *Diabetes* 51:2148-2157.

79. Ryan,E.A., Paty,B.W., Senior,P.A., Bigam,D., Alfadhli,E., Kneteman,N.M., Lakey,J.R., and Shapiro,A.M. 2005. Five-year follow-up after clinical islet transplantation. *Diabetes* 54:2060-2069.

80. Villiger,P., Ryan,E.A., Owen,R., O'Kelly,K., Oberholzer,J., Al Saif,F., Kin,T., Wang,H., Larsen,I., Blitz,S.L. *et al.* 2005. Prevention of bleeding after islet

transplantation: lessons learned from a multivariate analysis of 132 cases at a single institution. *Am.J.Transplant.* 5:2992-2998.

81. Molinari,M., Al Saif,F., Ryan,E.A., Lakey,J.R., Senior,P.A., Paty,B.W., Bigam,D.L., Kneteman,N.M., and Shapiro,A.M. 2005. Sirolimus-induced ulceration of the small bowel in islet transplant recipients: report of two cases. *Am.J.Transplant.* 5:2799-2804.

82. Senior,P.A., Paty,B.W., Cockfield,S.M., Ryan,E.A., and Shapiro,A.M. 2005. Proteinuria developing after clinical islet transplantation resolves with sirolimus withdrawal and increased tacrolimus dosing. *Am.J.Transplant.* 5:2318-2323.

83. Fabian,M.C., Lakey,J.R., Rajotte,R.V., and Kneteman,N.M. 1993. The efficacy and toxicity of rapamycin in murine islet transplantation. In vitro and in vivo studies. *Transplantation* 56:1137-1142.

84. BROWNING,H. and RESNIK,P. 1951. Homologous and heterologous transplantation of pancreatic tissue in normal and diabetic mice. *Yale J.Biol.Med.* 24:140-152.

85. BROOKS,J.R. and GIFFORD,G.H. 1959. Pancreatic homotransplantation. *Transplant.Bull.* 6:100-103.

86. BROOKS,J.R., STURGIS,S.H., and HILL,G.J. 1960. An evaluation of endocrine tissue homotransplantation in the millipore chamber: with a note on tissue adaptation to the host. *Ann.N.Y.Acad.Sci.* 87:482-500.

87. Kelly,W.D., Lillehei,R.C., Merkel,F.K., Idezuki,Y., and Goetz,F.C. 1967. Allotransplantation of the pancreas and duodenum along with the kidney in diabetic nephropathy. *Surgery* 61:827-837.

88. Bartlett,S.T., Schweitzer,E.J., Johnson,L.B., Kuo,P.C., Papadimitriou,J.C., Drachenberg,C.B., Klassen,D.K., Hoehn-Saric,E.W., Weir,M.R., and Imbembo,A.L. 1996. Equivalent success of simultaneous pancreas kidney and solitary pancreas transplantation. A prospective trial of tacrolimus immunosuppression with percutaneous biopsy. *Ann.Surg.* 224:440-449.

89. Gruessner,R.W., Sutherland,D.E., Najarian,J.S., Dunn,D.L., and Gruessner,A.C. 1997. Solitary pancreas transplantation for nonuremic patients with labile insulin-dependent diabetes mellitus. *Transplantation* 64:1572-1577.

90. Sutherland,D.E., Gruessner,R.W., Dunn,D.L., Matas,A.J., Humar,A., Kandaswamy,R., Mauer,S.M., Kennedy,W.R., Goetz,F.C., Robertson,R.P. *et al.* 2001. Lessons learned from more than 1,000 pancreas transplants at a single institution. *Ann.Surg.* 233:463-501.

91. Cohen,D.J., St Martin,L., Christensen,L.L., Bloom,R.D., and Sung,R.S. 2006. Kidney and pancreas transplantation in the United States, 1995-2004. *Am.J.Transplant.* 6:1153-1169.

92. Ryan,E.A., Bigam,D., and Shapiro,A.M. 2006. Current indications for pancreas or islet transplant. *Diabetes Obes.Metab* 8:1-7.

93. Calafiore,R. 1997. Perspectives in pancreatic and islet cell transplantation for the therapy of IDDM. *Diabetes Care* 20:889-896.

94. Humar,A., Kandaswamy,R., Granger,D., Gruessner,R.W., Gruessner,A.C., and Sutherland,D.E. 2000. Decreased surgical risks of pancreas transplantation in the modern era. *Ann.Surg.* 231:269-275.

95. Sutherland,D.E. 1994. Pancreas transplants. *Br.J.Surg.* 81:2-4.

96. Otonkoski,T., Gao,R., and Lundin,K. 2005. Stem cells in the treatment of diabetes. *Ann.Med.* 37:513-520.

97. Shi,Y., Hou,L., Tang,F., Jiang,W., Wang,P., Ding,M., and Deng,H. 2005. Inducing embryonic stem cells to differentiate into pancreatic beta cells by a novel three-step approach with activin A and all-trans retinoic acid. *Stem Cells* 23:656-662.

98. Segev,H., Fishman,B., Ziskind,A., Shulman,M., and Itskovitz-Eldor,J. 2004. Differentiation of human embryonic stem cells into insulin-producing clusters. *Stem Cells* 22:265-274.

99. Kim,D., Gu,Y., Ishii,M., Fujimiya,M., Qi,M., Nakamura,N., Yoshikawa,T., Sumi,S., and Inoue,K. 2003. In vivo functioning and transplantable mature pancreatic islet-like cell clusters differentiated from embryonic stem cell. *Pancreas* 27:e34-e41.

100. Banerjee,M. and Bhonde,R.R. 2003. Islet generation from intra islet precursor cells of diabetic pancreas: in vitro studies depicting in vivo differentiation. *JOP.* 4:137-145.

101. Nir,T. and Dor,Y. 2005. How to make pancreatic beta cells--prospects for cell therapy in diabetes. *Curr.Opin.Biotechnol.* 16:524-529.

102. Bonner-Weir,S. and Weir,G.C. 2005. New sources of pancreatic beta-cells. *Nat.Biotechnol.* 23:857-861.

103. Trucco,M. 2005. Regeneration of the pancreatic beta cell. *J.Clin.Invest* 115:5-12.

104. Heit,J.J. and Kim,S.K. 2004. Embryonic stem cells and islet replacement in diabetes mellitus. *Pediatr.Diabetes* 5 Suppl 2:5-15.

105. Burns,C.J., Persaud,S.J., and Jones,P.M. 2004. Stem cell therapy for diabetes: do we need to make beta cells? *J.Endocrinol.* 183:437-443.

106. Blyszczuk,P. and Wobus,A.M. 2004. Stem cells and pancreatic differentiation in vitro. *J.Biotechnol.* 113:3-13.

107. Hussain,M.A. and Theise,N.D. 2004. Stem-cell therapy for diabetes mellitus. *Lancet* 364:203-205.

108. Yamada,S. and Kojima,I. 2005. Regenerative medicine of the pancreatic beta cells. *J.Hepatobiliary.Pancreat.Surg.* 12:218-226.

109. Ramiya,V. and Schatz,D. 2004. Islet replacement vs. regeneration: hope or hype? *Pediatr.Diabetes* 5 Suppl 2:45-56.

110. Sutherland,D.E., Sibley,R., Xu,X.Z., Michael,A., Srikanta,A.M., Taub,F., Najarian,J., and Goetz,F.C. 1984. Twin-to-twin pancreas transplantation: reversal and reenactment of the pathogenesis of type I diabetes. *Trans.Assoc.Am.Physicians* 97:80-87.

111. Ogawa,N., List,J.F., Habener,J.F., and Maki,T. 2004. Cure of overt diabetes in NOD mice by transient treatment with anti-lymphocyte serum and exendin-4. *Diabetes* 53:1700-1705.

112. Suarez-Pinzon,W.L., Yan,Y., Power,R., Brand,S.J., and Rabinovitch,A. 2005. Combination therapy with epidermal growth factor and gastrin increases beta-cell mass and reverses hyperglycemia in diabetic NOD mice. *Diabetes* 54:2596-2601.

113. Suarez-Pinzon,W.L., Lakey,J.R., Brand,S.J., and Rabinovitch,A. 2005. Combination therapy with epidermal growth factor and gastrin induces neogenesis of human islet {beta}-cells from pancreatic duct cells and an increase in functional {beta}-cell mass. *J.Clin.Endocrinol.Metab* 90:3401-3409.

114. Kodama,S., Kuhtreiber,W., Fujimura,S., Dale,E.A., and Faustman,D.L. 2003. Islet regeneration during the reversal of autoimmune diabetes in NOD mice. *Science* 302:1223-1227.

115. Pileggi,A., Ricordi,C., Kenyon,N.S., Froud,T., Baidal,D.A., Kahn,A., Selvaggi,G., and Alejandro,R. 2004. Twenty years of clinical islet transplantation at the Diabetes Research Institute--University of Miami. *Clin.Transpl.*177-204.

116. Matsumoto,S., Noguchi,H., Yonekawa,Y., Okitsu,T., Iwanaga,Y., Liu,X., Nagata,H., Kobayashi,N., and Ricordi,C. 2006. Pancreatic islet transplantation for treating diabetes. *Expert.Opin.Biol.Ther.* 6:23-37.

117. Froud,T., Ricordi,C., Baidal,D.A., Hafiz,M.M., Ponte,G., Cure,P., Pileggi,A., Poggioli,R., Ichii,H., Khan,A. *et al.* 2005. Islet transplantation in type 1 diabetes

mellitus using cultured islets and steroid-free immunosuppression: Miami experience. *Am.J.Transplant.* 5:2037-2046.

118. Ricordi,C., Inverardi,L., Kenyon,N.S., Goss,J., Bertuzzi,F., and Alejandro,R. 2005. Requirements for success in clinical islet transplantation. *Transplantation* 79:1298-1300.

119. Boker,A., Rothenberg,L., Hernandez,C., Kenyon,N.S., Ricordi,C., and Alejandro,R. 2001. Human islet transplantation: update. *World J.Surg.* 25:481-486.

120. Bhargava,R., Senior,P.A., Ackerman,T.E., Ryan,E.A., Paty,B.W., Lakey,J.R., and Shapiro,A.M. 2004. Prevalence of hepatic steatosis after islet transplantation and its relation to graft function. *Diabetes* 53:1311-1317.

121. Johansson,H., Goto,M., Dufrane,D., Siegbahn,A., Elgue,G., Gianello,P., Korsgren,O., and Nilsson,B. 2006. Low molecular weight dextran sulfate: a strong candidate drug to block IBMIR in clinical islet transplantation. *Am.J.Transplant.* 6:305-312.

122. Goto,M., Groth,C.G., Nilsson,B., and Korsgren,O. 2004. Intraportal pig islet xenotransplantation into athymic mice as an in vivo model for the study of the instant blood-mediated inflammatory reaction. *Xenotransplantation.* 11:195-202.

123. Ozmen,L., Ekdahl,K.N., Elgue,G., Larsson,R., Korsgren,O., and Nilsson,B. 2002. 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.

124. Bennet,W., Groth,C.G., Larsson,R., Nilsson,B., and Korsgren,O. 2000. 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.

125. Rothstein,D.M. and Sayegh,M.H. 2003. T-cell costimulatory pathways in allograft rejection and tolerance. *Immunol.Rev.* 196:85-108.

126. Schwartz,R.H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science* 248:1349-1356.

127. Jenkins,M.K., Taylor,P.S., Norton,S.D., and Urdahl,K.B. 1991. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J.Immunol.* 147:2461-2466.

128. Clarkson,M.R. and Sayegh,M.H. 2005. T-cell costimulatory pathways in allograft rejection and tolerance. *Transplantation* 80:555-563.

129. Rayat,G.R., Rajotte,R.V., and Korbutt,G.S. 1999. Potential application of neonatal porcine islets as treatment for type 1 diabetes: a review. *Ann.N.Y.Acad.Sci.* 875:175-188.

130. Koulmanda,M., Laufer,T.M., Auchincloss,H., Jr., and Smith,R.N. 2004. Prolonged survival of fetal pig islet xenografts in mice lacking the capacity for an indirect response. *Xenotransplantation.* 11:525-530.

131. Rayat,G.R., Johnson,Z.A., Beilke,J.N., Korbutt,G.S., Rajotte,R.V., and Gill,R.G. 2003. The degree of phylogenetic disparity of islet grafts dictates the reliance on indirect CD4 T-cell antigen recognition for rejection. *Diabetes* 52:1433-1440.

132. Baldwin,W.M., III, Pruitt,S.K., Brauer,R.B., Daha,M.R., and Sanfilippo,F. 1995. Complement in organ transplantation. Contributions to inflammation, injury, and rejection. *Transplantation* 59:797-808.

133. Goldsby RA, Kindt TJ, Osbourne BA, and Kuby J. 2003. Immunology. W.H. Freeman, New York.

134. Grabstein,K.H., Waldschmidt,T.J., Finkelman,F.D., Hess,B.W., Alpert,A.R., Boiani,N.E., Namen,A.E., and Morrissey,P.J. 1993. Inhibition of murine B and T lymphopoiesis in vivo by an anti-interleukin 7 monoclonal antibody. *J.Exp.Med.* 178:257-264.

135. Rossini,A.A. 2004. Autoimmune diabetes and the circle of tolerance. *Diabetes* 53:267-275.

136. Pearson,T., Markees,T.G., Serreze,D.V., Pierce,M.A., Wicker,L.S., Peterson,L.B., Shultz,L.D., Mordes,J.P., Rossini,A.A., and Greiner,D.L. 2003. Islet cell autoimmunity and transplantation tolerance: two distinct mechanisms? *Ann.N.Y.Acad.Sci.* 1005:148-156.

137. Okitsu,T., Bartlett,S.T., Hadley,G.A., Drachenberg,C.B., and Farney,A.C. 2001. Recurrent autoimmunity accelerates destruction of minor and major histoincompatible islet grafts in nonobese diabetic (NOD) mice. *Am.J.Transplant.* 1:138-145.

138. Arreaza,G., Salojin,K., Yang,W., Zhang,J., Gill,B., Mi,Q.S., Gao,J.X., Meagher,C., Cameron,M., and Delovitch,T.L. 2003. Deficient activation and resistance to activation-induced apoptosis of CD8+ T cells is associated with defective peripheral tolerance in nonobese diabetic mice. *Clin.Immunol.* 107:103-115.

139. Decallonne,B., van Etten,E., Giulietti,A., Casteels,K., Overbergh,L., Bouillon,R., and Mathieu,C. 2003. Defect in activation-induced cell death in non-obese diabetic (NOD) T lymphocytes. *J.Autoimmun.* 20:219-226.

140. Noorchashm,H., Moore,D.J., Noto,L.E., Noorchashm,N., Reed,A.J., Reed,A.L., Song,H.K., Mozaffari,R., Jevnikar,A.M., Barker,C.F. *et al.* 2000. Impaired CD4 T cell activation due to reliance upon B cell-mediated costimulation in nonobese diabetic (NOD) mice. *J.Immunol.* 165:4685-4696.

141. Markees,T.G., Serreze,D.V., Phillips,N.E., Sorli,C.H., Gordon,E.J., Shultz,L.D., Noelle,R.J., Woda,B.A., Greiner,D.L., Mordes,J.P. *et al.* 1999. NOD mice have a generalized defect in their response to transplantation tolerance induction. *Diabetes* 48:967-974.

142. Shi,Q., Wang,D., Hadley,G.A., Bingaman,A.W., Bartlett,S.T., and Farber,D.L. 2004. Long-term islet graft survival in NOD mice by abrogation of recurrent autoimmunity. *Diabetes* 53:2338-2345.

143. Makhlouf,L., Grey,S.T., Dong,V., Csizmadia,E., Arvelo,M.B., Auchincloss,H., Jr., Ferran,C., and Sayegh,M.H. 2004. Depleting anti-CD4 monoclonal antibody cures new-onset diabetes, prevents recurrent autoimmune diabetes, and delays allograft rejection in nonobese diabetic mice. *Transplantation* 77:990-997.

144. Gordon,E.J., Wicker,L.S., Peterson,L.B., Serreze,D.V., Markees,T.G., Shultz,L.D., Rossini,A.A., Greiner,D.L., and Mordes,J.P. 2005. Autoimmune diabetes and resistance to xenograft transplantation tolerance in NOD mice. *Diabetes* 54:107-115.

145. Koulmanda,M., Qipo,A., Smith,R.N., and Auchincloss,H., Jr. 2003. Pig islet xenografts are resistant to autoimmune destruction by non-obese diabetic recipients after anti-CD4 treatment. *Xenotransplantation.* 10:178-184.

146. O'Connell,P. 2002. Pancreatic islet xenotransplantation. *Xenotransplantation.* 9:367-371.

147. Korbutt,G.S., Elliott,J.F., Ao,Z., Smith,D.K., Warnock,G.L., and Rajotte,R.V. 1996. Large scale isolation, growth, and function of porcine neonatal islet cells. *J.Clin.Invest* 97:2119-2129.

148. Phelps,C.J., Koike,C., Vaught,T.D., Boone,J., Wells,K.D., Chen,S.H., Ball,S., Specht,S.M., Polejaeva,I.A., Monahan,J.A. *et al.* 2003. Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science* 299:411-414.

149. Dor,F.J., Tseng,Y.L., Cheng,J., Moran,K., Sanderson,T.M., Lancos,C.J., Shimizu,A., Yamada,K., Awwad,M., Sachs,D.H. *et al.* 2004. alpha1,3-Galactosyltransferase gene-knockout miniature swine produce natural cytotoxic anti-Gal antibodies. *Transplantation* 78:15-20.

150. Boneva,R.S. and Folks,T.M. 2004. Xenotransplantation and risks of zoonotic infections. *Ann.Med.* 36:504-517.

151. Fishman,J.A. and Patience,C. 2004. Xenotransplantation: infectious risk revisited. *Am.J.Transplant.* 4:1383-1390.

152. Magre,S., Takeuchi,Y., and Bartosch,B. 2003. Xenotransplantation and pig endogenous retroviruses. *Rev.Med.Virol.* 13:311-329.

153. Cardona,K., Korbutt,G.S., Milas,Z., Lyon,J., Cano,J., Jiang,W., Bello-Laborn,H., Hacquoil,B., Strobert,E., Gangappa,S. *et al.* 2006. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat.Med.* 12:304-306.

154. Dorling,A. 2002. Clinical xenotransplantation: pigs might fly? *Am.J.Transplant.* 2:695-700.

155. Blusch,J.H., Patience,C., and Martin,U. 2002. Pig endogenous retroviruses and xenotransplantation. *Xenotransplantation.* 9:242-251.

156. Herring,C., Cunningham,D.A., Whittam,A.J., Fernandez-Suarez,X.M., and Langford,G.A. 2001. Monitoring xenotransplant recipients for infection by PERV. *Clin.Biochem.* 34:23-27.

157. Kobayashi,T., Harb,G., and Rayat,G.R. 2005. Prolonged survival of microencapsulated neonatal porcine islets in mice treated with a combination of anti-CD154 and anti-LFA-1 monoclonal antibodies. *Transplantation* 80:821-827.

158. Rayat,G.R. and Gill,R.G. 2005. Indefinite survival of neonatal porcine islet xenografts by simultaneous targeting of LFA-1 and CD154 or CD45RB. *Diabetes* 54:443-451.

159. Rayat,G.R., Rajotte,R.V., Ao,Z., and Korbutt,G.S. 2000. Microencapsulation of neonatal porcine islets: protection from human antibody/complement-mediated cytolysis in vitro and long-term reversal of diabetes in nude mice. *Transplantation* 69:1084-1090.

160. Kin,T., Korbutt,G.S., Kobayashi,T., Dufour,J.M., and Rajotte,R.V. 2005. Reversal of diabetes in pancreatectomized pigs after transplantation of neonatal porcine islets. *Diabetes* 54:1032-1039.

161. Appel,M.C., Banuelos,S.J., Greiner,D.L., Shultz,L.D., Mordes,J.P., and Rossini,A.A. 2004. Prolonged survival of neonatal porcine islet xenografts in mice treated with a donor-specific transfusion and anti-CD154 antibody. *Transplantation* 77:1341-1349.

162. Dufrane,D., Goebbels,R.M., Fdilat,I., Guiot,Y., and Gianello,P. 2005. Impact of porcine islet size on cellular structure and engraftment after transplantation: adult versus young pigs. *Pancreas* 30:138-147.

163. Komoda,H., Miyagawa,S., Omori,T., Takahagi,Y., Murakami,H., Shigehisa,T., Ito,T., Matsuda,H., and Shirakura,R. 2005. Survival of adult islet grafts from transgenic pigs with N-acetylglucosaminyltransferase-III (GnT-III) in cynomolgus monkeys. *Xenotransplantation.* 12:209-216.

164. Duvivier-Kali,V.F., Omer,A., Lopez-Avalos,M.D., O'Neil,J.J., and Weir,G.C. 2004. Survival of microencapsulated adult pig islets in mice in spite of an antibody response. *Am.J.Transplant.* 4:1991-2000.

165. Kirchhof,N., Shibata,S., Wijkstrom,M., Kulick,D.M., Salerno,C.T., Clemmings,S.M., Heremans,Y., Galili,U., Sutherland,D.E., Dalmasso,A.P. *et al.* 2004. Reversal of diabetes in non-immunosuppressed rhesus macaques by intraportal porcine islet xenografts precedes acute cellular rejection. *Xenotransplantation.* 11:396-407.

166. Maeda,A., Goto,M., Zhang,J., Bennet,W., Groth,C.G., Korsgren,O., and Wennberg,L. 2003. Immunosuppression with FTY720 and cyclosporine A inhibits rejection of adult porcine islet xenografts in rats. *Transplantation* 75:1409-1414.

167. Buhler,L., Deng,S., O'Neil,J., Kitamura,H., Koulmanda,M., Baldi,A., Rahier,J., Alwayn,I.P., Appel,J.Z., Awwad,M. *et al.* 2002. Adult porcine islet transplantation in baboons treated with conventional immunosuppression or a non-myeloablative regimen and CD154 blockade. *Xenotransplantation.* 9:3-13.

168. Cantarovich,D., Blancho,G., Potiron,N., Jugeau,N., Fiche,M., Chagneau,C., Letessier,E., Boeffard,F., Loth,P., Karam,G. *et al.* 2002. Rapid failure of pig islet transplantation in non human primates. *Xenotransplantation.* 9:25-35.

169. Wennberg,L., Song,Z., Bennet,W., Zhang,J., Nava,S., Sundberg,B., Bari,S., Groth,C.G., and Korsgren,O. 2001. Diabetic rats transplanted with adult porcine islets and immunosuppressed with cyclosporine A, mycophenolate mofetil, and leflunomide remain normoglycemic for up to 100 days. *Transplantation* 71:1024-1033.

170. Hering,B.J., Wijkstrom,M., Graham,M.L., Hardstedt,M., Aasheim,T.C., Jie,T., Ansite,J.D., Nakano,M., Cheng,J., Li,W. *et al.* 2006. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. *Nat.Med.* 12:301-303.

171. Bonner-Weir,S., Taneja,M., Weir,G.C., Tatarkiewicz,K., Song,K.H., Sharma,A., and O'Neil,J.J. 2000. In vitro cultivation of human islets from expanded ductal tissue. *Proc.Natl.Acad.Sci.U.S.A* 97:7999-8004.

172. Street,C.N., Lakey,J.R., Seeberger,K., Helms,L., Rajotte,R.V., Shapiro,A.M., and Korbutt,G.S. 2004. Heterogenous expression of nestin in human pancreatic tissue precludes its use as an islet precursor marker. *J.Endocrinol.* 180:213-225.

173. Rayat,G.R., Rajotte,R.V., Hering,B.J., Binette,T.M., and Korbutt,G.S. 2003. In vitro and in vivo expression of Galalpha-(1,3)Gal on porcine islet cells is age dependent. *J.Endocrinol.* 177:127-135.

174. Korsgren,O., Jansson,L., Eizirik,D., and Andersson,A. 1991. Functional and morphological differentiation of fetal porcine islet-like cell clusters after transplantation into nude mice. *Diabetologia* 34:379-386.

175. Asplund,K. 1973. Dynamics of insulin release from the foetal and neonatal rat pancreas. *Eur.J.Clin.Invest* 3:338-344.

176. Binette,T.M., Dufour,J.M., and Korbutt,G.S. 2001. 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.

177. Ezzelarab,M., Ayares,D., and Cooper,D.K. 2005. Carbohydrates in xenotransplantation. *Immunol.Cell Biol.* 83:396-404.

178. Kobayashi,T. and Cooper,D.K. 1999. Anti-Gal, alpha-Gal epitopes, and xenotransplantation. *Subcell.Biochem.* 32:229-257.

179. Kin,T., Korbutt,G.S., Kobayashi,T., Dufour,J.M., and Rajotte,R.V. 2005. Reversal of diabetes in pancreatectomized pigs after transplantation of neonatal porcine islets. *Diabetes* 54:1032-1039.

180. Appel,M.C., Banuelos,S.J., Greiner,D.L., Shultz,L.D., Mordes,J.P., and Rossini,A.A. 2004. Prolonged survival of neonatal porcine islet xenografts in mice treated with a donor-specific transfusion and anti-CD154 antibody. *Transplantation* 77:1341-1349.

181. Vizzardelli,C., Molano,R.D., Pileggi,A., Berney,T., Cattan,P., Fenjves,E.S., , Peel,A., Fraker,C., Ricordi,C., and Inverardi,L. 2002. Neonatal porcine pancreatic cell clusters as a potential source for transplantation in humans: characterization of proliferation, apoptosis, xenoantigen expression and gene delivery with recombinant AAV. *Xenotransplantation.* 9:14-24.

182. Korbutt,G.S., Ao,Z., Flashner,M., and Rajotte,R.V. 1997. Neonatal porcine islets as a possible source of tissue for humans and microencapsulation improves the metabolic response of islet graft posttransplantation. *Ann.N.Y.Acad.Sci.* 831:294-303.

183. Lukinius,A. and Korsgren,O. 2001. The transplanted fetal endocrine pancreas undergoes an inherent sequential differentiation similar to that in the native pancreas. An ultrastructural study in the pig-to-mouse model. *Diabetes* 50:962-971.

184. Otonkoski,T., Ustinov,J., Rasilainen,S., Kallio,E., Korsgren,O., and Hayry,P. 1999. Differentiation and maturation of porcine fetal islet cells in vitro and after transplantation. *Transplantation* 68:1674-1683.

185. Strokan,V., Bennet,W., Molne,J., Korsgren,O., and Breimer,M.E. 2000. Distribution of the Galalpha1-3Gal antigen in cultured adult and fetal porcine pancreatic islet cells: an immunoelectron microscopic study. *Transplantation* 70:846-851.

186. Bennet,W., Bjorkland,A., Sundberg,B., Davies,H., Liu,J., Holgersson,J., and Korsgren,O. 2000. A comparison of fetal and adult porcine islets with regard to Gal alpha (1,3)Gal expression and the role of human immunoglobulins and complement in islet cell cytotoxicity. *Transplantation* 69:1711-1717.

187. Reinholt,F.P., Hultenby,K., Tibell,A., Korsgren,O., and Groth,C.G. 1998. Survival of fetal porcine pancreatic islet tissue transplanted to a diabetic patient: findings by ultrastructural immunocytochemistry. *Xenotransplantation.* 5:222-225.

188. Groth,C.G., Korsgren,O., Tibell,A., Tollemar,J., Moller,E., Bolinder,J., Ostman,J., Reinholt,F.P., Hellerstrom,C., and Andersson,A. 1994. Transplantation of porcine fetal pancreas to diabetic patients. *Lancet* 344:1402-1404.

189. Asplund,K., Westman,S., and Hellerstrom,C. 1969. Glucose stimulation of insulin secretion from the isolated pancreas of foetal and newborn rats. *Diabetologia* 5:260-262.

190. Hole,R.L., Pian-Smith,M.C., and Sharp,G.W. 1988. Development of the biphasic response to glucose in fetal and neonatal rat pancreas. *Am.J.Physiol* 254:E167-E174.

191. Rhoten,W.B. 1980. Insulin secretory dynamics during development of rat pancreas. *Am.J.Physiol* 239:E57-E63.

192. Koulmanda,M., Qipo,A., Smith,R.N., and Auchincloss,H., Jr. 2003. Pig islet xenografts are resistant to autoimmune destruction by non-obese diabetic recipients after anti-CD4 treatment. *Xenotransplantation.* 10:178-184.

193. MacKenzie,D.A., Hullett,D.A., and Sollinger,H.W. 2003. Xenogeneic transplantation of porcine islets: an overview. *Transplantation* 76:887-891.

194. Tang,T.H., Li,C.L., Li,X., Jiang,F.Q., Zhang,Y.K., Ren,H.Q., Su,S.S., and Jiang,G.S. 2004. Immune tolerance in pancreatic islet xenotransplantation. *World J.Gastroenterol.* 10:1457-1461.

195. Takahashi,K., Rooney,A.P., and Nei,M. 2000. Origins and divergence times of mammalian class II MHC gene clusters. *J.Hered.* 91:198-204.

196. Cooper DKC, Kemp E, Platt JL, and White DJG. 1997. Xenotransplantation: The Transplantation of Organs and Tissues Between Species. Springer, New York.

197. Platt JL. 2001. Xenotransplantation. ASM Press, Washington, DC.

198. Tree of Life Web Project, Eutheria. Placental Mammals. The Tree of Life Web Project . 1997.
Ref Type: Electronic Citation

199. Borel,J.F., Baumann,G., Chapman,I., Donatsch,P., Fahr,A., Mueller,E.A., and Vigouret,J.M. 1996. In vivo pharmacological effects of ciclosporin and some analogues. *Adv.Pharmacol.* 35:115-246.

200. Rang HP, Dale MM, and Ritter JM. 1999. Pharmacology. Churchill Livingstone, Toronto.

201. Meier-Kriesche,H.U., Li,S., Gruessner,R.W., Fung,J.J., Bustami,R.T., Barr,M.L., and Leichtman,A.B. 2006. Immunosuppression: evolution in practice and trends, 1994-2004. *Am.J.Transplant.* 6:1111-1131.

202. Kaufman,D.B., Morel,P., Condie,R., Field,M.J., Rooney,M., Tzardis,P., Stock,P., and Sutherland,D.E. 1991. Beneficial and detrimental effects of RBC-adsorbed antilymphocyte globulin and prednisone on purified canine islet autograft and allograft function. *Transplantation* 51:37-42.

203. Rilo,H.L., Carroll,P.B., Zeng,Y.J., Fontes,P., Demetris,J., and Ricordi,C. 1994. Acceleration of chronic failure of intrahepatic canine islet autografts by a short course of prednisone. *Transplantation* 57:181-187.

204. Zeng,Y., Ricordi,C., Lendoire,J., Carroll,P.B., Alejandro,R., Bereiter,D.R., Tzakis,A., and Starzl,T.E. 1993. The effect of prednisone on pancreatic islet autografts in dogs. *Surgery* 113:98-102.

205. Nanji,S.A. and Shapiro,A.M. 2006. Advances in pancreatic islet transplantation in humans. *Diabetes Obes.Metab* 8:15-25.

206. Kroczek,R.A., Mages,H.W., and Hutloff,A. 2004. Emerging paradigms of T-cell co-stimulation. *Curr.Opin.Immunol.* 16:321-327.

207. Kapic,E., Becic,F., and Kusturica,J. 2004. Basiliximab, mechanism of action and pharmacological properties. *Med.Arh.* 58:373-376.

208. Bielekova,B., Catalfamo,M., Reichert-Scrivner,S., Packer,A., Cerna,M., Waldmann,T.A., McFarland,H., Henkart,P.A., and Martin,R. 2006. Regulatory CD56(bright) natural killer cells mediate immunomodulatory effects of IL-2Ralpha-targeted therapy (daclizumab) in multiple sclerosis. *Proc.Natl.Acad.Sci.U.S.A* 103:5941-5946.

209. Berthelot,C., Cather,J., Jones,D., and Duvic,M. 2006. Atypical CD8+ cutaneous T-cell lymphoma after immunomodulatory therapy. *Clin.Lymphoma Myeloma.* 6:329-332.

210. Berney,T., Pileggi,A., Molano,R.D., Poggioli,R., Zahr,E., Ricordi,C., and Inverardi,L. 2003. The effect of simultaneous CD154 and LFA-1 blockade on the survival of allogeneic islet grafts in nonobese diabetic mice. *Transplantation* 76:1669-1674.

211. Kircher,B., Latzer,K., Gastl,G., and Nachbaur,D. 2003. Comparative in vitro study of the immunomodulatory activity of humanized and chimeric anti-CD25 monoclonal antibodies. *Clin.Exp.Immunol.* 134:426-430.

212. Schulze-Koops,H., Burkhardt,H., and Kalden,J.R. 1999. What we have learned from trials of immunomodulatory agents in rheumatoid arthritis: Future directions. *Drugs Today (Barc.)* 35:327-351.

213. Gao,W., Demirci,G., Strom,T.B., and Li,X.C. 2003. Stimulating PD-1-negative signals concurrent with blocking CD154 co-stimulation induces long-term islet allograft survival. *Transplantation* 76:994-999.

214. Benda,B., Ljunggren,H.G., Peach,R., Sandberg,J.O., and Korsgren,O. 2002. Co-stimulatory molecules in islet xenotransplantation: CTLA4Ig treatment in CD40 ligand-deficient mice. *Cell Transplant.* 11:715-720.

215. Bucher,P., Gang,M., Morel,P., Mathe,Z., Bosco,D., Pernin,N., Wekerle,T., Berney,T., and Buhler,L.H. 2005. Transplantation of discordant xenogeneic islets using repeated therapy with anti-CD154. *Transplantation* 79:1545-1552.

216. Lehnert,A.M., Yi,S., Burgess,J.S., and O'Connell,P.J. 2000. Pancreatic islet xenograft tolerance after short-term costimulation blockade is associated with increased CD4+ T cell apoptosis but not immune deviation. *Transplantation* 69:1176-1185.

217. Mai,G., Bucher,P., Morel,P., Mei,J., Bosco,D., Andres,A., Mathe,Z., Wekerle,T., Berney,T., and Buhler,L.H. 2005. Anti-CD154 mAb treatment but not recipient CD154 deficiency leads to long-term survival of xenogeneic islet grafts. *Am.J.Transplant.* 5:1021-1031.

218. Lenschow,D.J., Zeng,Y., Thistlethwaite,J.R., Montag,A., Brady,W., Gibson,M.G., Linsley,P.S., and Bluestone,J.A. 1992. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science* 257:789-792.

219. Safley,S.A., Kapp,J.A., and Weber,C.J. 2002. Proliferative and cytokine responses in CTLA4-Ig-treated diabetic NOD mice transplanted with microencapsulated neonatal porcine ICCs. *Cell Transplant.* 11:695-705.

220. Lu,X., Schulz,M., Zihlmann,H.R., and Borel,J.F. 1998. Long-term survival of hamster islet xenografts in mice under short-course treatment with nondepleting versus depleting anti-CD4 monoclonal antibodies. *Xenotransplantation.* 5:154-163.

221. Sandrini,S. 2005. Use of IL-2 receptor antagonists to reduce delayed graft function following renal transplantation: a review. *Clin.Transplant.* 19:705-710.

222. Van Gelder,T., Warle,M., and Ter Meulen,R.G. 2004. Anti-interleukin-2 receptor antibodies in transplantation: what is the basis for choice? *Drugs* 64:1737-1741.

223. Swiatecka-Urban,A. 2003. Anti-interleukin-2 receptor antibodies for the prevention of rejection in pediatric renal transplant patients: current status. *Paediatr.Drugs* 5:699-716.

224. Ahsan,N. 2003. Induction immunotherapy with IL-2Ra monoclonal antibody in kidney transplantation. *Minerva Urol.Nefrol.* 55:67-79.

225. Waldmann,T.A. 2002. The IL-2/IL-15 receptor systems: targets for immunotherapy. *J.Clin.Immunol.* 22:51-56.

226. Anderson,C.C. 2006. Time, space and contextual models of the immunity tolerance decision: bridging the geographical divide of Zinkernagel and Hengartner's 'Credo 2004'. *Scand.J.Immunol.* 63:249-256.

227. Samstein,B. and Platt,J.L. 2001. Xenotransplantation and tolerance. *Philos.Trans.R.Soc.Lond B Biol.Sci.* 356:749-758.

228. Cosimi,A.B. and Sachs,D.H. 2004. Mixed chimerism and transplantation tolerance. *Transplantation* 77:943-946.

229. Wekerle,T. and Sykes,M. 2001. Mixed chimerism and transplantation tolerance. *Annu.Rev.Med.* 52:353-370.

230. Abe,M., Qi,J., Sykes,M., and Yang,Y.G. 2002. Mixed chimerism induces donor-specific T-cell tolerance across a highly disparate xenogeneic barrier. *Blood* 99:3823-3829.

231. Shi,Q., Wang,D., Hadley,G.A., Bingaman,A.W., Bartlett,S.T., and Farber,D.L. 2004. Long-term islet graft survival in NOD mice by abrogation of recurrent autoimmunity. *Diabetes* 53:2338-2345.

232. Li,H., Inverardi,L., Molano,R.D., Pileggi,A., and Ricordi,C. 2003. Nonlethal conditioning for the induction of allogeneic chimerism and tolerance to islet allografts. *Transplantation* 75:966-970.

233. Li,H., Abou el-Ezz,A.Y., Gambrell,B.E., and Ildstad,S.T. 1995. Mixed xenogeneic chimerism and tolerance III. A nonlethal approach to induce donor-

specific transplantation tolerance to simultaneous islet xenografts. *Transplantation* 60:59-65.

234. Neipp,M., Exner,B.G., and Ildstad,S.T. 1998. A nonlethal conditioning approach to achieve engraftment of xenogeneic rat bone marrow in mice and to induce donor-specific tolerance. *Transplantation* 66:969-975.

235. Sharabi,Y., Aksentijevich,I., Sundt,T.M., III, Sachs,D.H., and Sykes,M. 1990. Specific tolerance induction across a xenogeneic barrier: production of mixed rat/mouse lymphohematopoietic chimeras using a nonlethal preparative regimen. *J.Exp.Med.* 172:195-202.

236. Colson,Y.L., Xu,H., Huang,Y., and Ildstad,S.T. 2004. Mixed xenogeneic chimerism induces donor-specific humoral and cellular immune tolerance for cardiac xenografts. *J.Immunol.* 173:5827-5834.

237. Ohdan,H., Yang,Y.G., Shimizu,A., Swenson,K.G., and Sykes,M. 1999. Mixed chimerism induced without lethal conditioning prevents T cell- and anti-Gal alpha 1,3Gal-mediated graft rejection. *J.Clin.Invest* 104:281-290.

238. Liang,Y., Huang,T., Zhang,C., Todorov,I., Atkinson,M., Kandeel,F., Forman,S., and Zeng,D. 2005. Donor CD8+ T cells facilitate induction of chimerism and tolerance without GVHD in autoimmune NOD mice conditioned with anti-CD3 mAb. *Blood* 105:2180-2188.

239. Powell,J.D., Fitzhugh,C., Kang,E.M., Hsieh,M., Schwartz,R.H., and Tisdale,J.F. 2005. Low-dose radiation plus rapamycin promotes long-term bone marrow chimerism. *Transplantation* 80:1541-1545.

240. Baker,M.B., Altman,N.H., Podack,E.R., and Levy,R.B. 1996. The role of cell-mediated cytotoxicity in acute GVHD after MHC-matched allogeneic bone marrow transplantation in mice. *J.Exp.Med.* 183:2645-2656.

241. Wysocki,C.A., Panoskaltsis-Mortari,A., Blazar,B.R., and Serody,J.S. 2005. Leukocyte migration and graft-versus-host disease. *Blood* 105:4191-4199.

242. Blazar,B.R. and Murphy,W.J. 2005. Bone marrow transplantation and approaches to avoid graft-versus-host disease (GVHD). *Philos.Trans.R.Soc.Lond B Biol.Sci.* 360:1747-1767.

243. Gallegos,A.M. and Bevan,M.J. 2006. Central tolerance: good but imperfect. *Immunol.Rev.* 209:290-296.

244. Sprent,J. 2005. Proving negative selection in the thymus. *J.Immunol.* 174:3841-3842.

245. Levy,M.M., Ketchum,R.J., Tomaszewski,J.E., Naji,A., Barker,C.F., and Brayman,K.L. 2002. Intrathymic islet transplantation in the canine: I. Histological

and functional evidence of autologous intrathymic islet engraftment and survival in pancreatectomized recipients. *Transplantation* 73:842-852.

246. Oluwole,O.O., Depaz,H.A., Gopinathan,R., Ali,A., Garrovillo,M., Jin,M.X., Hardy,M.A., and Oluwole,S.F. 2001. Indirect allorecognition in acquired thymic tolerance: induction of donor-specific permanent acceptance of rat islets by adoptive transfer of allopeptide-pulsed host myeloid and thymic dendritic cells. *Diabetes* 50:1546-1552.

247. Turvey,S.E., Hara,M., Morris,P.J., and Wood,K.J. 1999. Mechanisms of tolerance induction after intrathymic islet injection: determination of the fate of alloreactive thymocytes. *Transplantation* 68:30-39.

248. Ali,A., Garrovillo,M., Jin,M.X., Hardy,M.A., and Oluwole,S.F. 2000. Major histocompatibility complex class I peptide-pulsed host dendritic cells induce antigen-specific acquired thymic tolerance to islet cells. *Transplantation* 69:221-226.

249. Saborio,D.V., Chowdhury,N.C., Jin,M.X., Chandraker,A., Sayegh,M.H., and Oluwole,S.F. 1999. Regulatory T cells maintain peripheral tolerance to islet allografts induced by intrathymic injection of MHC class I allopeptides. *Cell Transplant.* 8:375-381.

250. Wu,A., Yamada,K., Neville,D.M., Awwad,M., Wain,J.C., Shimizu,A., Gojo,S., Kitamura,H., Colvin,R.B., Cooper,D.K. *et al.* 2003. Xenogeneic thymus transplantation in a pig-to-baboon model. *Transplantation* 75:282-291.

251. Lee,L.A., Gritsch,H.A., Sergio,J.J., Arn,J.S., Glaser,R.M., Sablinski,T., Sachs,D.H., and Sykes,M. 1994. Specific tolerance across a discordant xenogeneic transplantation barrier. *Proc.Natl.Acad.Sci.U.S.A* 91:10864-10867.

252. Yamamoto,S., Lavelle,J.M., Vagefi,P.A., Arakawa,H., Samelson-Jones,E., Moran,S., Teranishi,K., Kamano,C., Fishman,J., Awwad,M. *et al.* 2005. Vascularized thymic lobe transplantation in a pig-to-baboon model: a novel strategy for xenogeneic tolerance induction and T-cell reconstitution. *Transplantation* 80:1783-1790.

253. Rodriguez-Barbosa,J.I., Zhao,Y., Zhao,G., Ezquerra,A., and Sykes,M. 2002. Murine CD4 T cells selected in a highly disparate xenogeneic porcine thymus graft do not show rapid decay in the absence of selecting MHC in the periphery. *J.Immunol.* 169:6697-6710.

254. Fehervari,Z. and Sakaguchi,S. 2004. CD4+ Tregs and immune control. *J.Clin.Invest* 114:1209-1217.

255. Jiang,H. and Chess,L. 2004. An integrated view of suppressor T cell subsets in immunoregulation. *J.Clin.Invest* 114:1198-1208.

256. Battaglia,M., Stabilini,A., Draghici,E., Gregori,S., Mocchetti,C., Bonifacio,E., and Roncarolo,M.G. 2006. Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance. *Diabetes* 55:40-49.

257. Gregori,S., Casorati,M., Amuchastegui,S., Smiroldo,S., Davalli,A.M., and Adorini,L. 2001. Regulatory T cells induced by 1 alpha,25-dihydroxyvitamin D3 and mycophenolate mofetil treatment mediate transplantation tolerance. *J.Immunol.* 167:1945-1953.

258. Ikemoto,T., Tashiro,S., Yasutomo,K., Kishihara,K., Kurita,N., and Miyake,H. 2004. Donor-specific tolerance induced by simultaneous allogeneic islet transplantation with CD4+CD25+ T-cells into hepatic parenchyma in mice. *J.Med.Invest* 51:178-185.

259. Nasr,I.W., Wang,Y., Gao,G., Deng,S., Diggs,L., Rothstein,D.M., Tellides,G., Lakkis,F.G., and Dai,Z. 2005. Testicular immune privilege promotes transplantation tolerance by altering the balance between memory and regulatory T cells. *J.Immunol.* 174:6161-6168.

260. Sanchez-Fueyo,A., Weber,M., Domenig,C., Strom,T.B., and Zheng,X.X. 2002. Tracking the immunoregulatory mechanisms active during allograft tolerance. *J.Immunol.* 168:2274-2281.

261. Porter,C.M. and Bloom,E.T. 2005. Human CD4+CD25+ regulatory T cells suppress anti-porcine xenogeneic responses. *Am.J.Transplant.* 5:2052-2057.

262. Colovai,A.I., Liu,Z., Ciubotariu,R., Lederman,S., Cortesini,R., and Suciu-Foca,N. 2000. Induction of xenoreactive CD4+ T-cell anergy by suppressor CD8+. *Transplantation* 69:1304-1310.

263. Lanza,R.P. and Chick,W.L. 1997. Transplantation of encapsulated cells and tissues. *Surgery* 121:1-9.

264. Lanza,R.P., Hayes,J.L., and Chick,W.L. 1996. Encapsulated cell technology. *Nat.Biotechnol.* 14:1107-1111.

265. Maki,T., Otsu,I., O'Neil,J.J., Dunleavy,K., Mullon,C.J., Solomon,B.A., and Monaco,A.P. 1996. Treatment of diabetes by xenogeneic islets without immunosuppression. Use of a vascularized bioartificial pancreas. *Diabetes* 45:342-347.

266. Lanza,R.P. and Chick,W.L. 1997. Immunoisolation: at a turning point. *Immunol.Today* 18:135-139.

267. Gray,D.W. 2001. An overview of the immune system with specific reference to membrane encapsulation and islet transplantation. *Ann.N.Y.Acad.Sci.* 944:226-239.

268. La Flamme,K.E., Mor,G., Gong,D., La Tempa,T., Fusaro,V.A., Grimes,C.A., and Desai,T.A. 2005. Nanoporous alumina capsules for cellular macroencapsulation: transport and biocompatibility. *Diabetes Technol.Ther.* 7:684-694.

269. Qi,M., Gu,Y., Sakata,N., Kim,D., Shirouzu,Y., Yamamoto,C., Hiura,A., Sumi,S., and Inoue,K. 2004. PVA hydrogel sheet macroencapsulation for the bioartificial pancreas. *Biomaterials* 25:5885-5892.

270. Isayeva,I.S., Kasibhatla,B.T., Rosenthal,K.S., and Kennedy,J.P. 2003. Characterization and performance of membranes designed for macroencapsulation/implantation of pancreatic islet cells. *Biomaterials* 24:3483-3491.

271. Lembert,N., Petersen,P., Wesche,J., Zschocke,P., Enderle,A., Doser,M., Planck,H., Becker,H.D., and Ammon,H.P. 2001. In vitro test of new biomaterials for the development of a bioartificial pancreas. *Ann.N.Y.Acad.Sci.* 944:271-276.

272. Suzuki,K., Bonner-Weir,S., Trivedi,N., Yoon,K.H., Hollister-Lock,J., Colton,C.K., and Weir,G.C. 1998. Function and survival of macroencapsulated syngeneic islets transplanted into streptozocin-diabetic mice. *Transplantation* 66:21-28.

273. Jesser,C., Kessler,L., Lambert,A., Belcourt,A., and Pinget,M. 1996. Pancreatic islet macroencapsulation: a new device for the evaluation of artificial membrane. *Artif.Organs* 20:997-1007.

274. Orive,G., Hernandez,R.M., Rodriguez,G.A., Calafiore,R., Chang,T.M., de Vos,P., Hortelano,G., Hunkeler,D., Lacik,I., and Pedraz,J.L. 2004. History, challenges and perspectives of cell microencapsulation. *Trends Biotechnol.* 22:87-92.

275. Orive,G., Gascon,A.R., Hernandez,R.M., Igartua,M., and Luis,P.J. 2003. Cell microencapsulation technology for biomedical purposes: novel insights and challenges. *Trends Pharmacol.Sci.* 24:207-210.

276. de Vos,P., Wolters,G.H., Fritschy,W.M., and Van Schilfgaarde,R. 1993. Obstacles in the application of microencapsulation in islet transplantation. *Int.J.Artif.Organs* 16:205-212.

277. Kobayashi,T., Aomatsu,Y., Iwata,H., Kin,T., Kanehiro,H., Hisanaga,M., Ko,S., Nagao,M., and Nakajima,Y. 2003. Indefinite islet protection from autoimmune destruction in nonobese diabetic mice by agarose microencapsulation without immunosuppression. *Transplantation* 75:619-625.

278. Duvivier-Kali,V.F., Omer,A., Parent,R.J., O'Neil,J.J., and Weir,G.C. 2001. Complete protection of islets against allorejection and autoimmunity by a simple barium-alginate membrane. *Diabetes* 50:1698-1705.

279. Dickson,B.C., Yang,H., Savelkoul,H.F., Rowden,G., van Rooijen,N., and Wright,J.R., Jr. 2003. Islet transplantation in the discordant tilapia-to-mouse model: a novel application of alginate microencapsulation in the study of xenograft rejection. *Transplantation* 75:599-606.

280. Sato,H., Kobayasi,T., Murakami,M., Kimura,T., Yamaguchi,A., Nakagawara,G., and Iwata,H. 2002. Improving function and survival of porcine islet xenografts using microencapsulation and culture preconditioning. *Pancreas* 25:e42-e49.

281. Kobayashi,T., Harb,G., and Rayat,G.R. 2005. Prolonged survival of microencapsulated neonatal porcine islets in mice treated with a combination of anti-CD154 and anti-LFA-1 monoclonal antibodies. *Transplantation* 80:821-827.

282. Fritschy,W.M., Wolters,G.H., and Van Schilfgaarde,R. 1991. Effect of alginate-polylysine-alginate microencapsulation on in vitro insulin release from rat pancreatic islets. *Diabetes* 40:37-43.

283. de Vos,P., De Haan,B., Wolters,G.H., and Van Schilfgaarde,R. 1996. Factors influencing the adequacy of microencapsulation of rat pancreatic islets. *Transplantation* 62:888-893.

284. de Vos,P., De Haan,B., Pater,J., and Van Schilfgaarde,R. 1996. Association between capsule diameter, adequacy of encapsulation, and survival of microencapsulated rat islet allografts. *Transplantation* 62:893-899.

285. Korbutt,G.S., Elliott,J.F., and Rajotte,R.V. 1997. Cotransplantation of allogeneic islets with allogeneic testicular cell aggregates allows long-term graft survival without systemic immunosuppression. *Diabetes* 46:317-322.

286. Selawry,H.P. and Cameron,D.F. 1993. Sertoli cell-enriched fractions in successful islet cell transplantation. *Cell Transplant.* 2:123-129.

287. Bellgrau,D., Gold,D., Selawry,H., Moore,J., Franzusoff,A., and Duke,R.C. 1995. A role for CD95 ligand in preventing graft rejection. *Nature* 377:630-632.

288. Cupp,A.S., Kim,G., and Skinner,M.K. 1999. Expression and action of transforming growth factor beta (TGFbeta1, TGFbeta2, and TGFbeta3) during embryonic rat testis development. *Biol.Reprod.* 60:1304-1313.

289. Bailey,R. and Griswold,M.D. 1999. Clusterin in the male reproductive system: localization and possible function. *Mol.Cell Endocrinol.* 151:17-23.

290. Griffith,T.S., Brunner,T., Fletcher,S.M., Green,D.R., and Ferguson,T.A. 1995. Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* 270:1189-1192.

291. Merly,F., Huard,C., Asselin,I., Robbins,P.D., and Tremblay,J.P. 1998. Anti-inflammatory effect of transforming growth factor-beta1 in myoblast transplantation. *Transplantation* 65:793-799.

292. Wahl,S.M., McCartney-Francis,N., and Mergenhagen,S.E. 1989. Inflammatory and immunomodulatory roles of TGF-beta. *Immunol.Today* 10:258-261.

293. Jenne,D.E. and Tschopp,J. 1989. Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: identity to sulfated glycoprotein 2, a constituent of rat testis fluid. *Proc.Natl.Acad.Sci.U.S.A* 86:7123-7127.

294. Nagata,S. and Golstein,P. 1995. The Fas death factor. *Science* 267:1449-1456.

295. Alderson,M.R., Tough,T.W., Davis-Smith,T., Braddy,S., Falk,B., Schooley,K.A., Goodwin,R.G., Smith,C.A., Ramsdell,F., and Lynch,D.H. 1995. Fas ligand mediates activation-induced cell death in human T lymphocytes. *J.Exp.Med.* 181:71-77.

296. Chen,W. and Wahl,S.M. 2003. TGF-beta: the missing link in CD4+CD25+ regulatory T cell-mediated immunosuppression. *Cytokine Growth Factor Rev.* 14:85-89.

297. Huber,S. and Schramm,C. 2006. TGF-beta and CD4+CD25+ regulatory T cells. *Front Biosci.* 11:1014-1023.

298. Aoki,C.A., Borchers,A.T., Li,M., Flavell,R.A., Bowlus,C.L., Ansari,A.A., and Gershwin,M.E. 2005. Transforming growth factor beta (TGF-beta) and autoimmunity. *Autoimmun.Rev.* 4:450-459.

299. Huang,S.S. and Huang,J.S. 2005. TGF-beta control of cell proliferation. *J.Cell Biochem.* 96:447-462.

300. Wahl,S.M., Swisher,J., McCartney-Francis,N., and Chen,W. 2004. TGF-beta: the perpetrator of immune suppression by regulatory T cells and suicidal T cells. *J.Leukoc.Biol.* 76:15-24.

301. Bommireddy,R. and Doetschman,T. 2004. TGF-beta, T-cell tolerance and anti-CD3 therapy. *Trends Mol.Med.* 10:3-9.

302. Wahl,S.M. and Chen,W. 2003. TGF-beta: how tolerant can it be? *Immunol.Res.* 28:167-179.

303. Dufour,J.M., Rajotte,R.V., Korbutt,G.S., and Emerich,D.F. 2003. Harnessing the immunomodulatory properties of Sertoli cells to enable xenotransplantation in type I diabetes. *Immunol.Invest* 32:275-297.

304. Dufour,J.M., Rajotte,R.V., Kin,T., and Korbutt,G.S. 2003. Immunoprotection of rat islet xenografts by cotransplantation with sertoli cells and a single injection of antilymphocyte serum. *Transplantation* 75:1594-1596.

305. Yang,H., Al Jazaeri,A., and Wright,J.R., Jr. 2002. The immunoprotective effect of Sertoli cells coencapsulated with islet xenografts is not dependent upon Fas ligand expression. *Cell Transplant.* 11:799-801.

306. Luca,G., Calvitti,M., Neri,L.M., Becchetti,E., Capitani,S., Basta,G., Angeletti,G., Fanelli,C., Brunetti,P., and Calafiore,R. 2000. Sertoli cell-induced reversal of adult rat pancreatic islet beta-cells into fetal-like status: potential implications for islet transplantation in type I diabetes mellitus. *J.Investig.Med.* 48:441-448.

307. Suarez-Pinzon,W., Korbutt,G.S., Power,R., Hooton,J., Rajotte,R.V., and Rabinovitch,A. 2000. 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.

308. Korbutt,G.S., Suarez-Pinzon,W.L., Power,R.F., Rajotte,R.V., and Rabinovitch,A. 2000. Testicular Sertoli cells exert both protective and destructive effects on syngeneic islet grafts in non-obese diabetic mice. *Diabetologia* 43:474-480.

309. Yang,H. and Wright,J.R., Jr. 1999. Co-encapsulation of Sertoli enriched testicular cell fractions further prolongs fish-to-mouse islet xenograft survival. *Transplantation* 67:815-820.

310. Kin,T., Rajotte,R.V., Dufour,J.M., and Korbutt,G.S. 2002. Development of an immunoprivileged site to prolong islet allograft survival. *Cell Transplant.* 11:547-552.

311. Selawry,H.P., Whittington,K.B., and Bellgrau,D. 1989. Abdominal intratesticular islet-xenograft survival in rats. *Diabetes* 38 Suppl 1:220-223.

312. Selawry,H.P. and Whittington,K.B. 1988. Prolonged intratesticular islet allograft survival is not dependent on local steroidogenesis. *Horm.Metab Res.* 20:562-565.

313. Abraham,R.T. and Wiederrecht,G.J. 1996. Immunopharmacology of rapamycin. *Annu.Rev.Immunol.* 14:483-510.

314. Vezina,C., Kudelski,A., and Sehgal,S.N. 1975. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J.Antibiot.(Tokyo)* 28:721-726.

315. Sehgal,S.N., Baker,H., and Vezina,C. 1975. Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. *J.Antibiot.(Tokyo)* 28:727-732.

316. Dumont,F.J. and Su,Q. 1996. Mechanism of action of the immunosuppressant rapamycin. *Life Sci.* 58:373-395.

317. Martel,R.R., Klicius,J., and Galet,S. 1977. Inhibition of the immune response by rapamycin, a new antifungal antibiotic. *Can.J.Physiol Pharmacol.* 55:48-51.

318. Raught,B., Gingras,A.C., and Sonenberg,N. 2001. The target of rapamycin (TOR) proteins. *Proc.Natl.Acad.Sci.U.S.A* 98:7037-7044.

319. Zhang,H., Stallock,J.P., Ng,J.C., Reinhard,C., and Neufeld,T.P. 2000. Regulation of cellular growth by the Drosophila target of rapamycin dTOR. *Genes Dev.* 14:2712-2724.

320. Loewith,R., Jacinto,E., Wullschleger,S., Lorberg,A., Crespo,J.L., Bonenfant,D., Oppliger,W., Jenoe,P., and Hall,M.N. 2002. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol.Cell* 10:457-468.

321. Long,X., Spycher,C., Han,Z.S., Rose,A.M., Muller,F., and Avruch,J. 2002. TOR deficiency in C. elegans causes developmental arrest and intestinal atrophy by inhibition of mRNA translation. *Curr.Biol.* 12:1448-1461.

322. Menand,B., Desnos,T., Nussaume,L., Berger,F., Bouchez,D., Meyer,C., and Robaglia,C. 2002. Expression and disruption of the Arabidopsis TOR (target of rapamycin) gene. *Proc.Natl.Acad.Sci.U.S.A* 99:6422-6427.

323. Cruz,M.C., Cavallo,L.M., Gorlach,J.M., Cox,G., Perfect,J.R., Cardenas,M.E., and Heitman,J. 1999. Rapamycin antifungal action is mediated via conserved complexes with FKBP12 and TOR kinase homologs in Cryptococcus neoformans. *Mol.Cell Biol.* 19:4101-4112.

324. Rowinsky,E.K. 2004. Targeting the molecular target of rapamycin (mTOR). *Curr.Opin.Oncol.* 16:564-575.

325. Kwon,G., Marshall,C.A., Pappan,K.L., Remedi,M.S., and McDaniel,M.L. 2004. Signaling elements involved in the metabolic regulation of mTOR by nutrients, incretins, and growth factors in islets. *Diabetes* 53 Suppl 3:S225-S232.

326. Inoki,K., Ouyang,H., Li,Y., and Guan,K.L. 2005. Signaling by target of rapamycin proteins in cell growth control. *Microbiol.Mol.Biol.Rev.* 69:79-100.

327. Crespo,J.L. and Hall,M.N. 2002. Elucidating TOR signaling and rapamycin action: lessons from Saccharomyces cerevisiae. *Microbiol.Mol.Biol.Rev.* 66:579-91, table.

328. Yatscoff,R.W., Wang,P., Chan,K., Hicks,D., and Zimmerman,J. 1995. Rapamycin: distribution, pharmacokinetics, and therapeutic range investigations. *Ther.Drug Monit.* 17:666-671.

329. Karyekar,C.S., Pradhan,R.S., Freeney,T., Ji,Q., Edeki,T., Chiu,W., Awni,W.M., Locke,C., Schwartz,L.B., Granneman,R.G. *et al.* 2005. A phase I multiple-dose escalation study characterizing pharmacokinetics and safety of ABT-578 in healthy subjects. *J.Clin.Pharmacol.* 45:910-918.

330. Gross,J. 2006. Rapamycin (Sirolimus): A two-edged sword? *J.Mol.Cell Cardiol.*

331. Vlahakis,N.E., Rickman,O.B., and Morgenthaler,T. 2004. Sirolimus-associated diffuse alveolar hemorrhage. *Mayo Clin.Proc.* 79:541-545.

332. Zakliczynski,M., Nozynski,J., Zakliczynska,H., Rozentryt,P., and Zembala,M. 2003. Deterioration of renal function after replacement of cyclosporine with sirolimus in five patients with severe renal impairment late after heart transplantation. *Transplant.Proc.* 35:2331-2332.

333. Montalbano,M., Neff,G.W., Yamashiki,N., Meyer,D., Bettiol,M., Slapak-Green,G., Ruiz,P., Manten,E., Safdar,K., O'Brien,C. *et al.* 2004. A retrospective review of liver transplant patients treated with sirolimus from a single center: an analysis of sirolimus-related complications. *Transplantation* 78:264-268.

334. Van Gelder,T., ter Meulen,C.G., Hene,R., Weimar,W., and Hoitsma,A. 2003. Oral ulcers in kidney transplant recipients treated with sirolimus and mycophenolate mofetil. *Transplantation* 75:788-791.

335. Ryan,E.A., Paty,B.W., Senior,P.A., Bigam,D., Alfadhli,E., Kneteman,N.M., Lakey,J.R., and Shapiro,A.M. 2005. Five-year follow-up after clinical islet transplantation. *Diabetes* 54:2060-2069.

336. Schampaert,E., Moses,J.W., Schofer,J., Schluter,M., Gershlick,A.H., Cohen,E.A., Palisaitis,D.A., Breithardt,G., Donohoe,D.J., Wang,H. *et al.* 2006. Sirolimus-Eluting Stents at Two Years: A Pooled Analysis of SIRIUS, E-SIRIUS, and C-SIRIUS With Emphasis on Late Revascularizations and Stent Thromboses. *Am.J.Cardiol.* 98:36-41.

337. Georgakis,G.V. and Younes,A. 2006. From Rapa Nui to rapamycin: targeting PI3K/Akt/mTOR for cancer therapy. *Expert.Rev.Anticancer Ther.* 6:131-140.

338. Giles,F.J. and Albitar,M. 2005. Mammalian target of rapamycin as a therapeutic target in leukemia. *Curr.Mol.Med.* 5:653-661.

339. Meric-Bernstam,F. and Esteva,F.J. 2005. Potential role of mammalian target of rapamycin inhibitors in breast cancer therapy. *Clin.Breast Cancer* 6:357-360.

340. Gomez-Martin,C., Rubio-Viqueira,B., and Hidalgo,M. 2005. Current status of mammalian target of rapamycin inhibitors in lung cancer. *Clin.Lung Cancer* 7 Suppl 1:S13-S18.

341. Ringshausen,I., Peschel,C., and Decker,T. 2005. Mammalian target of rapamycin (mTOR) inhibition in chronic lymphocytic B-cell leukemia: a new therapeutic option. *Leuk.Lymphoma* 46:11-19.

342. Anderson,K.M. and Zimring,J.C. 2006. Rapamycin prolongs susceptibility of responding T cells to tolerance induction by CD8+ veto cells. *Transplantation* 81:88-94.

343. Battaglia,M., Stabilini,A., Draghici,E., Gregori,S., Mocchetti,C., Bonifacio,E., and Roncarolo,M.G. 2006. Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance. *Diabetes* 55:40-49.

344. Battaglia,M., Stabilini,A., Draghici,E., Migliavacca,B., Gregori,S., Bonifacio,E., and Roncarolo,M.G. 2006. Induction of Tolerance in Type 1 Diabetes via Both CD4+CD25+ T Regulatory Cells and T Regulatory Type 1 Cells. *Diabetes* 55:1571-1580.

345. Battaglia,M., Stabilini,A., and Roncarolo,M.G. 2005. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 105:4743-4748.

346. Davignon,D., Martz,E., Reynolds,T., Kurzinger,K., and Springer,T.A. 1981. Lymphocyte function-associated antigen 1 (LFA-1): a surface antigen distinct from Lyt-2,3 that participates in T lymphocyte-mediated killing. *Proc.Natl.Acad.Sci.U.S.A* 78:4535-4539.

347. Dougherty,G.J. and Hogg,N. 1987. The role of monocyte lymphocyte function-associated antigen 1 (LFA-1) in accessory cell function. *Eur.J.Immunol.* 17:943-947.

348. Godwin,J.W., d'Apice,A.J., and Cowan,P.J. 2004. Characterization of pig intercellular adhesion molecule-2 and its interaction with human LFA-1. *Am.J.Transplant.* 4:515-525.

349. Van Epps,D.E., Potter,J., Vachula,M., Smith,C.W., and Anderson,D.C. 1989. Suppression of human lymphocyte chemotaxis and transendothelial migration by anti-LFA-1 antibody. *J.Immunol.* 143:3207-3210.

350. Herold,K.C., Vezys,V., Gage,A., and Montag,A.G. 1994. Prevention of autoimmune diabetes by treatment with anti-LFA-1 and anti-ICAM-1 monoclonal antibodies. *Cell Immunol.* 157:489-500.

351. Milicevic,N.M., Nohroudi,K., Milicevic,Z., and Westermann,J. 2004. 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.

352. Shimizu,Y. 2003. LFA-1: more than just T cell Velcro. *Nat.Immunol.* 4:1052-1054.

353. Carrasco,Y.R., Fleire,S.J., Cameron,T., Dustin,M.L., and Batista,F.D. 2004. LFA-1/ICAM-1 interaction lowers the threshold of B cell activation by facilitating B cell adhesion and synapse formation. *Immunity.* 20:589-599.

354. Perez,O.D., Mitchell,D., Jager,G.C., South,S., Murriel,C., McBride,J., Herzenberg,L.A., Kinoshita,S., and Nolan,G.P. 2003. 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.

355. Ni,H.T., Deeths,M.J., Li,W., Mueller,D.L., and Mescher,M.F. 1999. Signaling pathways activated by leukocyte function-associated Ag-1-dependent costimulation. *J.Immunol.* 162:5183-5189.

356. Nicolls,M.R. and Gill,R.G. 2006. LFA-1 (CD11a) as a therapeutic target. *Am.J.Transplant.* 6:27-36.

357. Shamri,R., Grabovsky,V., Gauguet,J.M., Feigelson,S., Manevich,E., Kolanus,W., Robinson,M.K., Staunton,D.E., von Andrian,U.H., and Alon,R. 2005. Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nat.Immunol.* 6:497-506.

358. Chesnutt,B.C., Smith,D.F., Raffler,N.A., Smith,M.L., White,E.J., and Ley,K. 2006. Induction of LFA-1-dependent neutrophil rolling on ICAM-1 by engagement of E-selectin. *Microcirculation.* 13:99-109.

359. Atarashi,K., Hirata,T., Matsumoto,M., Kanemitsu,N., and Miyasaka,M. 2005. Rolling of Th1 cells via P-selectin glycoprotein ligand-1 stimulates LFA-1-mediated cell binding to ICAM-1. *J.Immunol.* 174:1424-1432.

360. Mueller,K.L., Daniels,M.A., Felthauser,A., Kao,C., Jameson,S.C., and Shimizu,Y. 2004. Cutting edge: LFA-1 integrin-dependent T cell adhesion is regulated by both ag specificity and sensitivity. *J.Immunol.* 173:2222-2226.

361. Hogg,N., Smith,A., McDowall,A., Giles,K., Stanley,P., Laschinger,M., and Henderson,R. 2004. How T cells use LFA-1 to attach and migrate. *Immunol.Lett.* 92:51-54.

362. Jungheim,K., Caspar,G., Usadel,K.H., and Schumm-Draeger,P.M. 2004. Lymphocyte homing in xenotransplanted human thyroid tissue can be inhibited by LFA-1 and ICAM-1 antibodies. *Thyroid* 14:3-11.

363. Borthwick,N.J., Akbar,A.A., Buckley,C., Pilling,D., Salmon,M., Jewell,A.P., and Yong,K.L. 2003. Transendothelial migration confers a survival advantage to

activated T lymphocytes: role of LFA-1/ICAM-1 interactions. *Clin.Exp.Immunol.* 134:246-252.

364. Jun,C.D., Shimaoka,M., Carman,C.V., Takagi,J., and Springer,T.A. 2001. Dimerization and the effectiveness of ICAM-1 in mediating LFA-1-dependent adhesion. *Proc.Natl.Acad.Sci.U.S.A* 98:6830-6835.

365. Barber,D.F., Faure,M., and Long,E.O. 2004. LFA-1 contributes an early signal for NK cell cytotoxicity. *J.Immunol.* 173:3653-3659.

366. Nicolls,M.R., Coulombe,M., Beilke,J., Gelhaus,H.C., and Gill,R.G. 2002. CD4-dependent generation of dominant transplantation tolerance induced by simultaneous perturbation of CD154 and LFA-1 pathways. *J.Immunol.* 169:4831-4839.

367. Larsson,L.C., Corbascio,M., Widner,H., Pearson,T.C., Larsen,C.P., and Ekberg,H. 2002. Simultaneous inhibition of B7 and LFA-1 signaling prevents rejection of discordant neural xenografts in mice lacking CD40L. *Xenotransplantation.* 9:68-76.

368. Bertry-Coussot,L., Lucas,B., Danel,C., Halbwachs-Mecarelli,L., Bach,J.F., Chatenoud,L., and Lemarchand,P. 2002. Long-term reversal of established autoimmunity upon transient blockade of the LFA-1/intercellular adhesion molecule-1 pathway. *J.Immunol.* 168:3641-3648.

369. Nicolls,M.R., Coulombe,M., Yang,H., Bolwerk,A., and Gill,R.G. 2000. Anti-LFA-1 therapy induces long-term islet allograft acceptance in the absence of IFN-gamma or IL-4. *J.Immunol.* 164:3627-3634.

370. Lebwohl,M., Tyring,S.K., Hamilton,T.K., Toth,D., Glazer,S., Tawfik,N.H., Walicke,P., Dummer,W., Wang,X., Garovoy,M.R. *et al.* 2003. A novel targeted T-cell modulator, efalizumab, for plaque psoriasis. *N.Engl.J.Med.* 349:2004-2013.

CHAPTER 2

RAPAMCYIN AND ANTI-LFA-1 PREVENT THE REJECTION OF RAT ISLET XENOGRAFTS IN BALB/C MICE

2.1 INTRODUCTION

Type 1 diabetes mellitus (T1DM) is a chronic metabolic disorder characterized by the autoimmune destruction of the insulin producing beta cells of the pancreas. The success of the Edmonton Protocol has once again put islet transplantation in the spotlight as a true alternative for the treatment of T1DM (1). Unfortunately, at present, widespread clinical application of this treatment is limited by the shortage of donor cadaveric pancreases. The scope of the clinical application of this therapy is further limited by the need for continuous immunosuppressive therapy, which is associated with a number of harmful side effects (2-5). The focus of our research is to develop a tolerizing regimen which is given short term, and is capable of sustaining long term acceptance of transplanted islet grafts, thereby circumventing the need for continuous immunosuppression.

Rapamycin (sirolimus) an immunosuppressive drug, is one of the mainstays of immunosuppressive therapy in the Edmonton Protocol (5). Rapamycin inhibits the proliferation of T and B lymphocytes by binding to its intracellular target, the mammalian target of rapamycin (mTOR). mTOR has a crucial role in the regulation of protein synthesis and translation initiation. When rapamycin binds to mTOR, it inhibits mTOR,

thereby preventing protein synthesis and arresting the cell in the G1 phase of the cell cycle (6-9).

The use of monoclonal antibodies to prevent the immune rejection of transplanted tissue is quickly becoming vital component of therapy in many different types of transplantation (10-13). Monoclonal antibodies can be used to deplete or inhibit specific components of the immune system or target cell-surface adhesion molecules (14;15). One monoclonal antibody of particular interest is anti-leukocyte function associated antigen-1 (anti-LFA-1). LFA-1 (CD11a/CD18) is a member of a family of structurally and functionally similar leukocyte differentiation antigens. LFA-1 is involved in the homing, adhesion and activation of lymphocytes. Antibodies directed towards LFA-1 have been shown to be beneficial in preventing transplanted graft rejection (16-21). Recently, a humanized form of anti-LFA-1, Efalizumab was approved for phase IV trials for the treatment of psoriasis (22).

Porcine islets have been proposed as a suitable alternative source of islets in clinical transplantation (23-26). However, at present many experimental models involving porcine tissue utilize mice as recipients, which represents a discordant relationship (27). If porcine islets are to become a source of islets in transplantation, the immune response must be examined as the relation between donor and recipient becomes more concordant. It has been well established that the indirect pathway of antigen presentation dominates in discordant transplantation, and that the direct pathway is increasingly active in the rejection of allogeneic and syngeneic transplants (28;29). Therefore, examining the value of potential clinical therapies to prevent xenograft

rejection and their ability to prevent concordant and discordant rejection is of significant importance.

In our study, we examined the efficacy of a short-term therapy with rapamycin and anti-LFA-1 in preventing the rejection of Wistar-Furth rat islets in BALB/c, a concordant donor/recipient relationship in an effort to further elucidate the mechanisms of concordant xenograft rejection and determine effective anti-rejection therapies.

2.2 MATERIALS AND METHODS

2.2.1 EXPERIMENTAL ANIMALS

Inbred male BALB/cByJ (BALB/c, H-2^d) were purchased from the Jackson Laboratory (Bar Harbor, ME) and Wistar-Furth (WF, $RT1^U$) rats were purchased from Harlan Laboratories (Indianapolis, IN). Recipient BALB/c mice were rendered diabetic by a single intraperitoneal injection of streptozotocin (250 mg/kg body weight; Sigma-Aldrich, Oakville, ON) 4 to 5 days before transplantation. All diabetic recipients had two consecutive non-fasting blood glucose levels above 17 mmol/l. The blood glucose of each recipient mouse was monitored by taking blood samples from the tail vein. Glucose levels were measured using a OneTouch Ultra glucometer (LifeScan, Burnaby, BC). All mice and rats were housed under pathogen-free conditions and fed standard laboratory food and water *ad libitum*, and cared for according to the guidelines established by the Health Sciences Animal Policy and Welfare Committee.

2.2.2 *IN VITRO* PROLIFERATION ASSAYS

Single cell suspensions of lymph node and spleen cells were prepared by mechanical disruption of lymph nodes and spleen between glass slides. Red blood cells from the spleen were depleted by incubation in red cell lysing buffer for 4 min. Mixed lymphocyte cultures were established by combining $5x10^5$/well responder cells (BALB/c lymph node) with $1x10^6$/ well irradiated (2.5 krad) spleen cells from WF rats. Triplicate cultures in a total volume of 0.2 ml of EMEM (Invitrogen, Burlington, ON) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen, Burlington, ON), $1x10^{-5}$M 2-mercaptoethanol, L-glutamine (Invitrogen, Burlington, ON) and 1% (v/v) antibiotics were established in 96-well flat-bottom plates at 37°C in 5% CO_2 in air. T cell proliferation was detected by pulsing cells of primary culture with 1μCi [^{3}H] thymidine for 22 hours on the indicated day of culture. Cells were harvested onto glass microfiber filters, (Wallac, Turku, Finland) and proliferation of cultures was quantified using a Wallac beta emission counter (Woodbridge, ON).

2.2.3 ISLET ISOLATION AND TRANSPLANTATION

WF rat pancreatic islets were isolated via distension of the pancreas, followed by collagenase (Sigma, Oakville, ON) digestion, density gradient centrifugation with dextran (Sigma, Oakville, ON) and handpicking of the islets. Islets were cultured overnight in Hams F10 media at 37°C and 5% CO_2. A total of 500 isolated rat islets were transplanted under the left kidney capsule of diabetic BALB/c mice as previously described (30). Engraftment was considered successful when the blood glucose level was ≤10 mM. Graft rejection was defined as the first of consecutive days of hyperglycemia (≥12 mM

glucose) and rejection was confirmed by histological analysis of the graft. Nephrectomy of the graft-bearing kidney was performed on a subset of recipients with long-term graft function (>100 days post-transplant) for the confirmation of graft-dependent euglycemia.

2.2.4 ANTI-REJECTION THERAPIES

Transplant recipients were randomly designated to receive the following anti-rejection treatments intraperitoneally: (1) rapamycin alone (0.2 mg/kg on days 0 thru 14 post-transplantation; a generous gift from Dr. James Shapiro, Edmonton, AB), (2) anti-LFA-1 monoclonal antibody alone (KBA; rat IgG2a; hybridoma kindly provided by Dr. Ron Gill, Denver, CO; 200μg on days 0, 1, 7, 14, 21 and 28 post-transplant), (3) rapamycin plus anti-LFA-1 monoclonal antibody. Untreated transplanted recipients served as the control for this experiment.

2.2.5 IMMUNOHISTOCHEMICAL STAINING

Four μm-thick sections of formalin-fixed tissues were stained to determine the presence of insulin containing cells. Insulin containing cells were detected by applying guinea-pig anti-porcine insulin primary antibody (1:1000; DAKO Corporation, Carprinteria, CA) for 30 min, followed by the addition of biotinylated goat anti-guinea pig IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA). The avidin-biotin complex/horseradish peroxidase (ABC/HP; Vector Laboratories) and 3, 3-diaminobenzidinetetrahydrochloride (DAB; BioGenex, San Ramon, CA) was used to produce a brown color. All sections were counterstained with Harris' hematoxylin and eosin.

Subpopulations of T cells (CD4 and CD8), granulocytes, macrophages and natural killer cells (CD11b) were also determined on tissues immersed in OCT compound and snap frozen at –80° C. Five μm sections of tissue were air dried for 10 min then fixed in acetone for 3 min at 4°C. These sections were then washed in PBS and non-specific binding was eliminated by incubating the tissue section in 2% FBS/PBS for 20 min. In addition, endogenous biotin or biotin-binding proteins present in the section were also eliminated using the avidin/biotin blocking kit (Vector Laboratories, Inc., Burlington, ON). Rat anti-mouse CD4 antibody (1:100; BD Pharmingen, Mississauga, ON) or rat anti-mouse CD8 antibody (1:100; BD Pharmingen, Mississauga, ON) or rat anti-mouse CD11b (1:500; BD Pharmingen, Mississauga, ON) was applied to each section for 30 min at room temperature. Biotinylated rabbit anti-rat IgG secondary antibody (1:200; Vector Laboratories, Inc. Burlington, ON) was added and incubated for 20 min. Avidin-Biotin Complex/Horseradish Peroxidase (ABC/HP) Reagent (Vector Laboratories, Inc. Burlington, ON) was applied, and 3, 3-diaminobenzidinetetrahydrochloride (DAB; BioGenex, San Ramon, CA) was used to produce a brown color. Sections were counterstained with Harris' hematoxylin.

2.2.6 SERUM ANTIBODY MEASUREMENT

To determine the effect of rapamycin and/or anti-LFA-1 monoclonal antibody on the production of mouse anti-rat antibodies, we determined the levels of mouse anti-rat IgG antibodies from the blood serum of each transplant recipient using flow cytometry. Peripheral blood samples from BALB/c mice were collected at the time of rejection or following long-term islet xenograft survival. The serum was isolated by centrifugation at

10000rpm for 10min. Rat spleen cells ($1x10^6$) obtained from the same rat islet donors were incubated with mouse serum at 1/128 dilution for 1 hour at 37°C (5% CO_2, 95% air). Spleen cells were then washed with PBS and incubated with FITC-conjugated goat anti-mouse IgG (rat adsorbed, 1:100; Southern Biotechnology Associates, Inc.) for 1 hour at 4°C. The percentage of cells bound to antibody was detected from single-parameter fluorescence histograms on a BD FACSCalibur (BD Bioscience, ON) after gating on viable lymphocytes. Controls for this experiment include sera from non-transplanted naïve BALB/c mice, unstained rat spleen cells, and rat spleen cells stained with secondary antibody alone without mouse serum.

2.2.7 STATISTICAL ANALYSIS

Statistical differences in graft survival among groups were analyzed using the Kaplan-Meier Log rank test. A p value of less than 0.05 was considered to be statistically significant.

2.3 RESULTS

2.3.1 TREATMENT WITH RAPAMCYIN AND ANTI-LFA-1 PREVENTS THE PROLIFERATION OF LYMPHOCYTES *IN VITRO*

BALB/c mouse lymphocytes were incubated with Wistar-Furth rat spleen cells in the presence of rapamycin, anti-LFA-1 or both. A marked reduction in proliferation as determined by [^{3}H] thymidine incorporation was observed in groups that received rapamycin, anti-LFA-1 or both (Figure 2-1). In comparison to the positive control (ConA), treatment with rapamycin or anti-LFA-1 almost completely abrogated any proliferation. This effect was independent of the vehicle of either anti-LFA-1 or

rapamycin. The additive effect of combining the two treatments on the prevention of proliferation of the mouse lymphocytes was small. Any combination of therapy appeared effective at inhibiting the proliferation of the lymphocytes *in vitro* regardless of the time point examined.

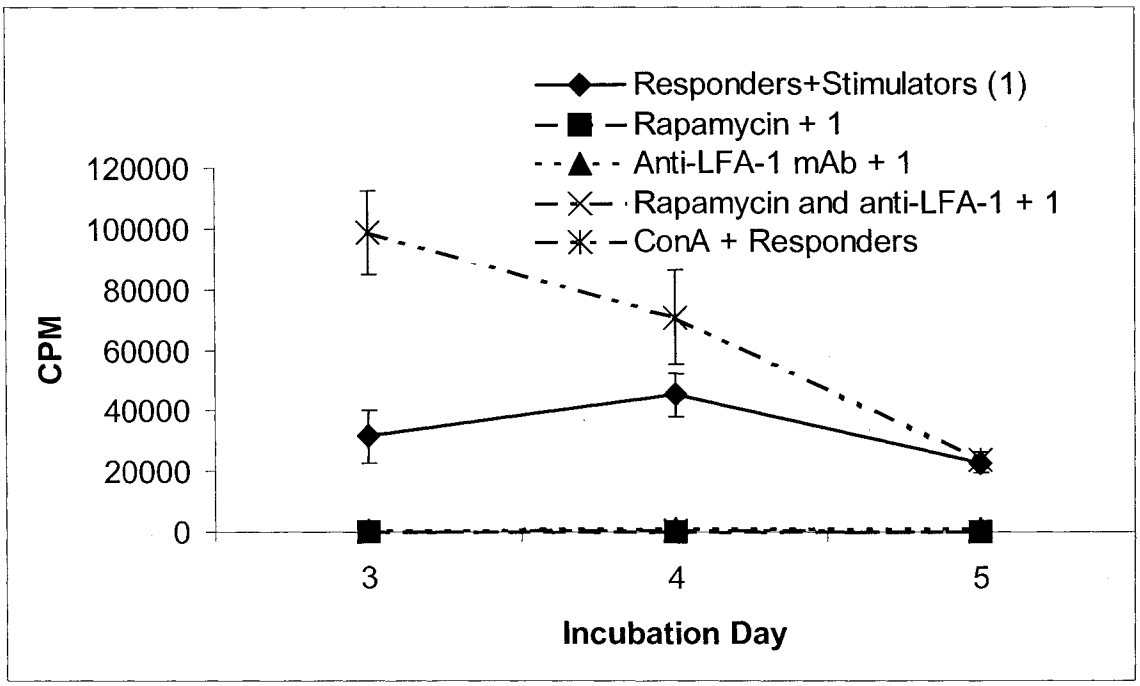


Figure 2-1. *In vitro* proliferation of BALB/c lymphocytes following stimulation with Wistar-Furth rat spleen cells. BALB/c lymphocytes (responders) were incubated with irradiated Wistar-Furth rat spleen cells (stimulators) for 3, 4, or 5 days. Following incubation, [^{3}H] thymidine was added to each well for 22 hours before the cells were harvested. Non-specific mitogen stimulation with ConA served as a positive control. Data is represented as the average count per minute of triplicate wells. A representative of 4 individual experiments is shown. Error bars represent the standard deviation.

2.3.2 RAPAMYCIN AND ANTI-LFA-1 PREVENT THE REJECTION OF RAT ISLET XENOGRAFTS

All recipients achieved normoglycemia following transplantation of 500 Wistar-Furth islets. Untreated recipients rapidly rejected their islet grafts, however in mice treated with rapamycin alone there was a modest prolongation of islet xenograft survival, but by day 80 all recipients had rejected their grafts ($P<0.05$ vs untreated) (Figure 2-2). In recipients treated with anti-LFA-1 alone there was a significant prolongation of islet graft survival and 97 percent of recipients' maintained normoglycemia for over 100 days ($P<0.05$ vs untreated). When anti-LFA-1 treatment was combined with rapamycin, islet graft protection increased and 99 percent of recipients maintained normoglycemia for over 100 days ($P<0.05$ vs untreated) (Table 2-1). A small group (3) of C57BL/6 mice were also transplanted with WF rat islets and treated with rapamycin and anti-LFA-1. All mice (3/3) maintained normoglycemia for over 100 days. Of mice that achieved long-term normoglycemia, a sample population was randomly selected and a survival nephrectomy was performed on the graft bearing kidney. All nephrectomized mice rapidly returned to hyperglycemia.

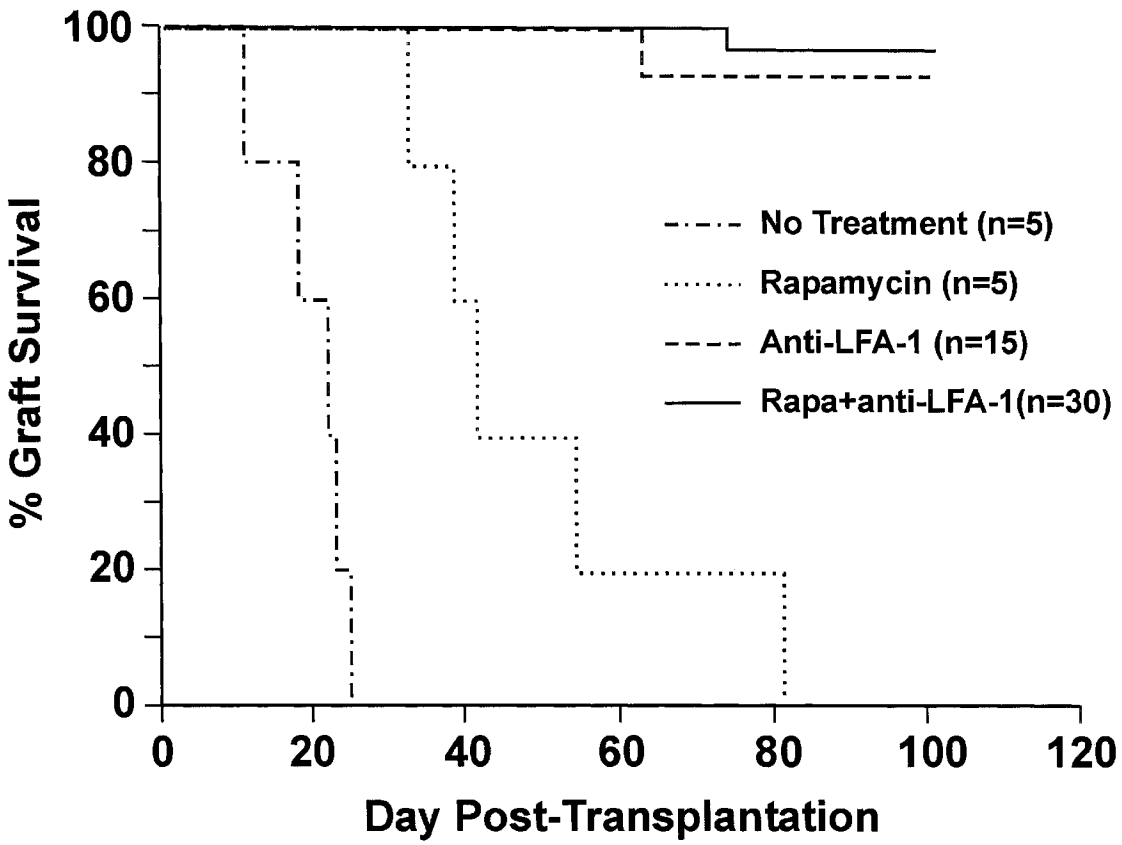


Figure 2-2. Treatment with anti-LFA-1 alone or in conjunction with rapamycin promotes long-term rat islet xenograft survival in BALB/c, but not with rapamycin treatment alone. Data represented as the percentage of graft survival in mice over the course of transplantation.

Treatment	n	Graft Survival (Days)	MST ± SD
No Treatment	5	10, 17, 21, 22, 24,	18.8 ± 5.5
Rapamycin alone	5	32, 38, 41, 54, 81	49.2 ± 19.5
Anti-LFA-1 alone	15	62, >100, >100, >100, >100, >100, >100, >100, >100, >100, >100, >100, >100, >100, >100,	97.5 ± 9.8
Rapamycin + Anti-LFA-1	30	73, >100	99.1 ± 4.9

Table 2-1. Graft survival in BALB/c mice transplanted with Wistar-Furth rat islets. Mice received no treatment, rapamycin alone, anti-LFA-1 antibody alone, or rapamycin in combination with anti-LFA-1. Mean graft survival time (MST) is reported in days post-transplantation ± standard deviation.

2.3.3 RAPAMYCIN AND ANTI-LFA-1 TREATMENT IS ASSOCIATED WITH A REDUCTION IN ISLET GRAFT INFILTRATION

All grafts were collected either following rejection or after 100 days for histologic examination of the islet graft and identification of cellular infiltrate. Grafts of untreated mice lacked insulin positive staining, however a large influx of mononuclear cells was present at the site of the graft (Figure 2-3). In rapamycin treated mice, virtually no insulin staining could be detected and again a large influx of cells was present within the graft. Anti-LFA-1 alone was successful in promoting long-term islet graft survival and was associated with a significant increase in the amount of insulin positive staining. However, treatment with anti-LFA-1 alone was not successful in preventing the influx of cells to the site of islet grafts, and at least some infiltrate could be detected. Grafts from mice treated with the combination of rapamycin and anti-LFA-1 contained robust insulin staining. This combination treatment was also associated a profound decrease in cellular infiltrate, although this finding was not absolute. In an effort to characterize the infiltrating cells present in the graft we performed CD4, CD8 and CD11b staining to detect the presence of $CD4^+$ T helper, $CD8^+$ T cells, and monocytes/macrophages/ granulocytes respectively. Significant $CD4^+$ staining was present in the grafts of untreated mice and this finding was also consistent with the grafts of rapamycin alone treated mice (Figure 2-4). $CD4^+$ T cells were also present in the grafts of anti-LFA-1 alone treated mice although to a lesser extent compared to the untreated and rapamycin treated groups. $CD8^+$ T cells were present in the grafts of islet recipients, albeit to a much lower extent in all recipient treatment groups. Consistent with the length of graft survival, the groups that received no treatment or rapamycin alone, a greater amount of $CD8^+$ T cells could be detected in the grafts. Treatment with anti-LFA-1 abrogated the

extent of $CD8^+$ T cell infiltration within the graft. Untreated mice or treatment with rapamycin alone did not prevent the recruitment of $CD11b^+$ cells to the graft site. However, treatment with anti-LFA-1 greatly reduced the extent of $CD11b^+$ cellular infiltration.

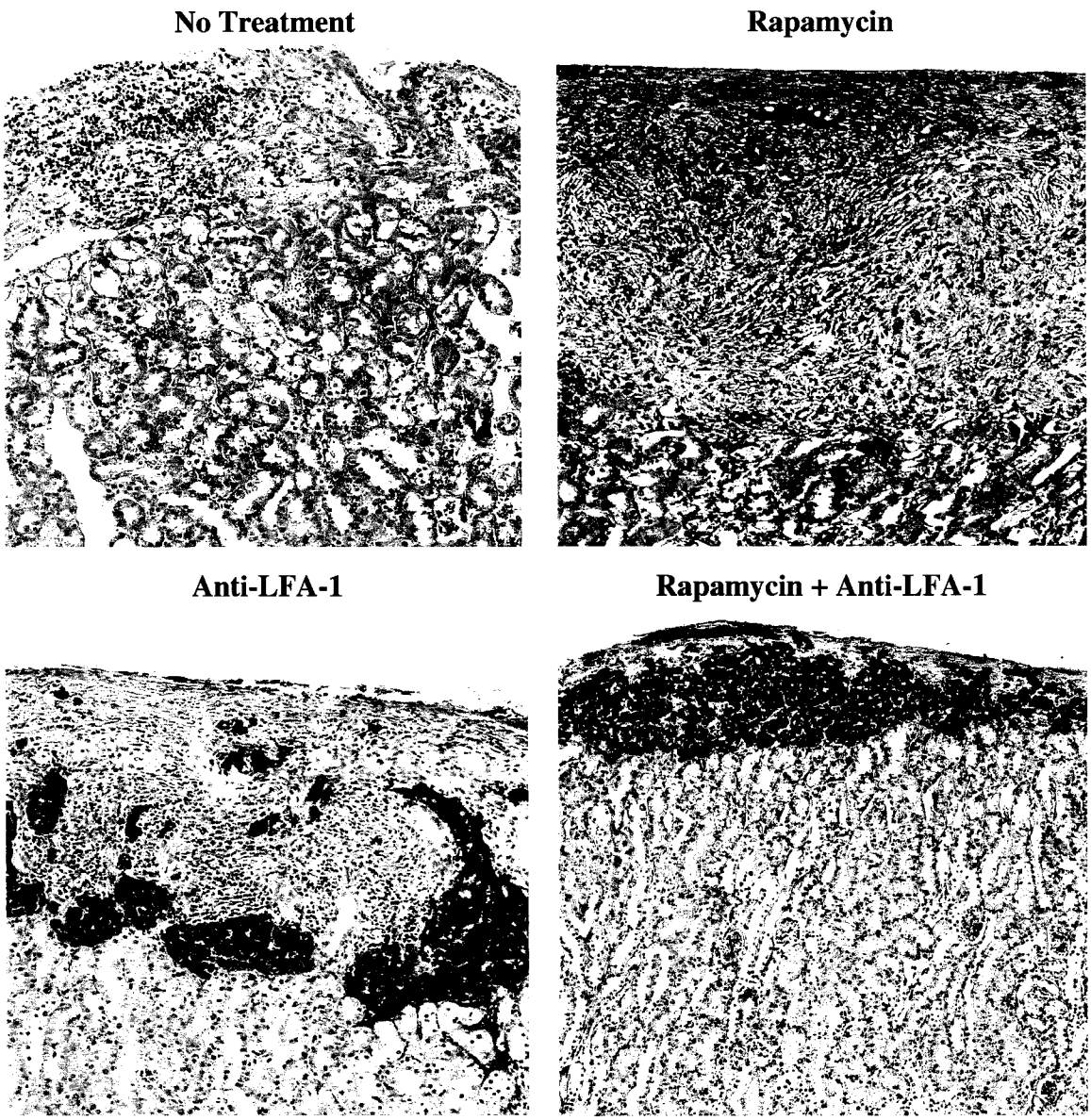


Figure 2-3. Insulin staining and histologic examination of the xenografts at the time of rejection or >100 days post-transplantation. Islet grafts retrieved from untreated recipients showed virtually no insulin positive cells (brown stain) and a large infiltration of mononuclear cells. Dispersed insulin positive staining was detected in the grafts from rapamycin treated mice and a large cellular influx. Extensive insulin positive staining as

well as a large cellular influx was present in anti-LFA-1 treated recipients. Rapamycin and anti-LFA-1 combination therapy was associated with robust insulin positive staining and a marked reduction in the infiltration of the islet graft. (Magnification 100x)

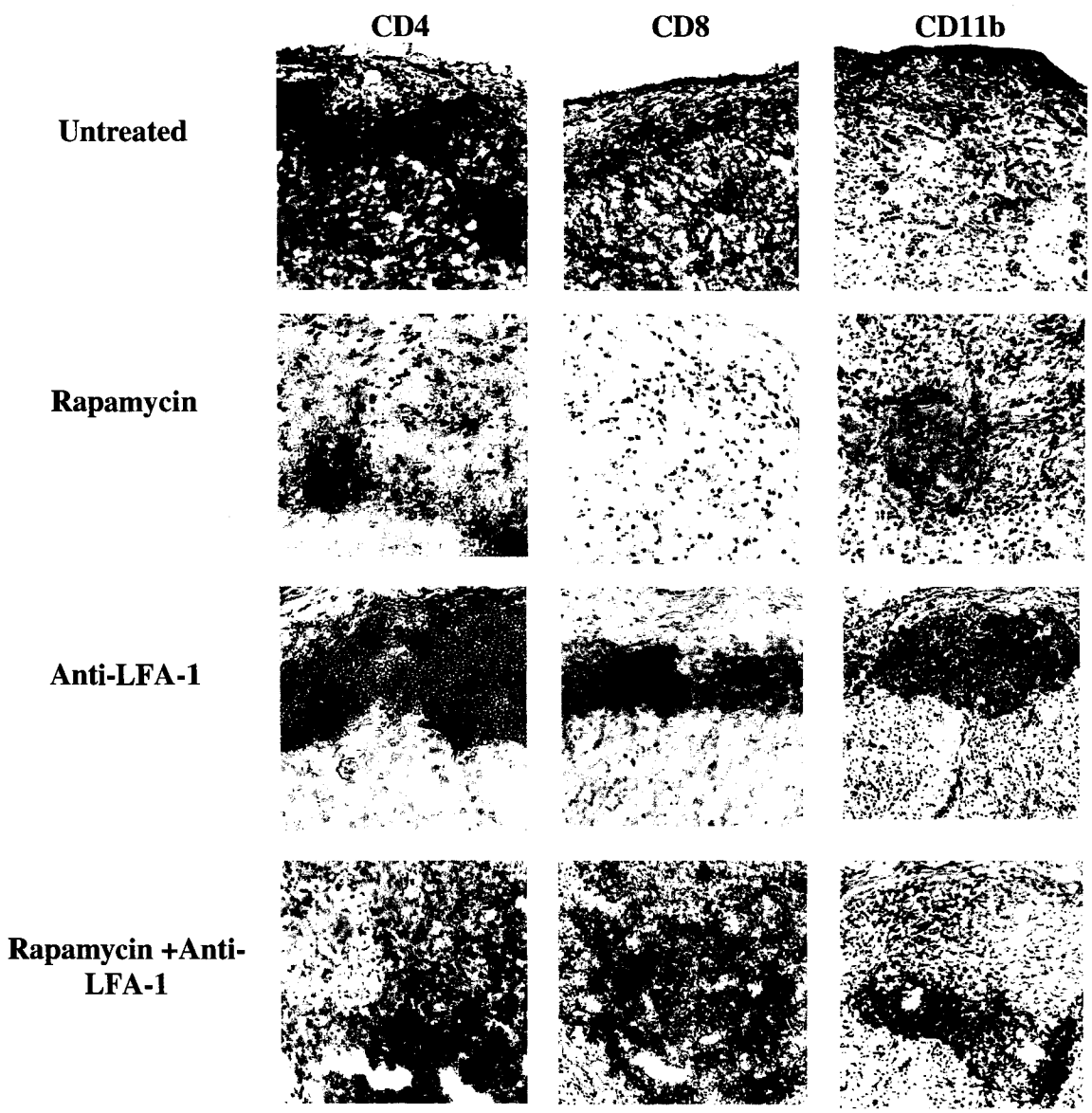


Figure 2-4. Immunohistochemical analysis of islet grafts from various treatment groups, for characterization of infiltrating mononuclear cell types. Staining for CD4 ($CD4^+$ T cells), CD8 ($CD8^+$ T cells) and CD11b (monocytes/macrophages, NK and granulocytes) was performed to identify these populations of cells within the islet grafts. Positive cells are indicated by brown staining. (Magnification 100x)

2.3.4 RAPAMYCIN AND ANTI-LFA-1 TREATMENT IS ASSOCIATED WITH A REDUCTION IN THE LEVEL OF MOUSE ANTI-RAT ANTIBODY

Recipient sera was analyzed using flow cytometry to determine the effect of the various treatments on the production of mouse anti-rat antibody levels. In mice that received no treatment, there was a significant shift of the peak compared to the naïve untransplanted control, indicating that significant production of mouse anti-rat antibody had occurred (Figure 2-5). Treatment with rapamycin decreased the production of antibodies slightly compared to the untreated group, however large amounts of mouse anti-rat antibodies could still be detected. In contrast, treatment with anti-LFA-1 alone or in conjunction with rapamycin resulted in a significant decrease in the production of mouse anti-rat antibodies to levels that were similar to the untransplanted control antibody levels.

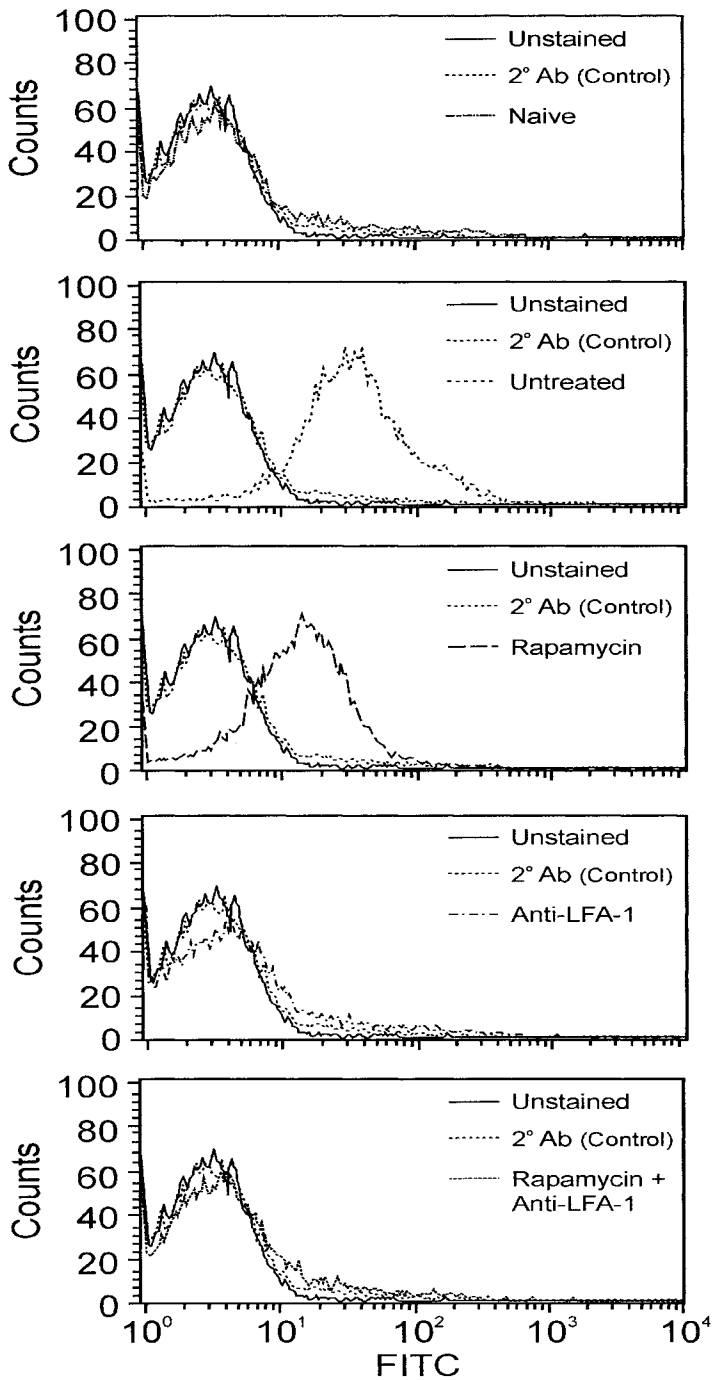


Figure 2-5. Analysis of mouse anti-rat antibodies in untreated and treated recipients using flow cytometry. A large production of mouse anti-rat antibodies was observed in the serum of untreated mice as indicated by the shift in the histogram peak. Treatment with rapamycin slightly decreased the production of anti-rat antibodies in these recipient mice. Treatment with anti-LFA-1 alone or in combination with rapamycin almost completely abrogated the production of mouse anti-rat antibodies.

2.4 DISCUSSION

The aim of the present study was to determine the efficacy of rapamycin and anti-LFA-1 treatment on the survival of rat islets transplanted to BALB/c mice. Rapamycin's beneficial effect in preventing the rejection of allo-islet grafts has been clearly demonstrated clinically (5). However, rapamycin's effectiveness in xenogeneic islet transplantation is not well established. LFA-1 has been identified as a major factor in islet xenograft rejection by previous studies (20;31). Therefore it was our objective to determine the effectiveness of a short-term therapy combining a general immunosuppressant (rapamycin) with a monoclonal antibody (anti-LFA-1) in preventing the rejection of a xenogeneic islet graft.

Initially, we performed mixed lymphocyte reaction (MLR) assays to determine the effect of rapamycin, anti-LFA-1 or a combination of both had on the proliferation of lymphocytes *in vitro*. In wells that received either rapamycin, anti-LFA-1 or both there was a marked decrease in proliferation of the lymphocytes as indicated by a decrease in the counts per minute (CPM). This was in contrast to the untreated wells or the ConA positive control, which exhibited a robust proliferative response. The inhibitory effect of either rapamycin or anti-LFA-1 was independent of the respective administration vehicles. These results indicate that treatment with either drug or in combination markedly suppressed a potential immune response *in vitro* at all time points examined.

We then examined the effect of rapamycin and anti-LFA-1 treatment *in vivo* and discovered that the combination of the two drugs resulted in long term (>100 days) islet xenograft survival in over 99% of recipients (29 of 30 mice). Treatment with rapamycin, prolonged islet graft survival, however, this treatment did not result in long-term graft

survival in any of the recipients. Anti-LFA-1 alone alternatively, was successful in promoting long-term islet xenograft survival in over 97% (14 of 15 mice) of recipients, indicating the majority of the beneficial effect of the combination therapy is attributed to anti-LFA-1 therapy. In addition, a small group of C57BL/6 mice were transplanted with Wistar-Furth rat islets, to determine if the benefit of this combination therapy was limited to BALB/c mice only. All of the C57BL/6 mice receiving Wistar-Furth rat islets and treated with the combination therapy maintained normoglycemia for over 100 days, indicating that the benefit of this therapy was not strain specific (data not shown). The constructive effect of the combination therapy appeared to be the most significant in the prevention of infiltration of the islet grafts by mononuclear cells. While some infiltrate could be detected in the combination treated mice grafts, the majority of the grafts were virtually free of cellular infiltrate and robust insulin staining could still be detected following 100 days post transplantation in all combination treated grafts. Conversely, mice that had rejected their grafts, large influxes of $CD4^+$ and $CD11b^+$ cells were detected in the graft, indicating the importance of helper T cells and macrophages respectively, in concordant xenograft rejection. In addition, treatment with the combination therapy almost completely abrogated the production of anti-rat antibodies in recipient mice. Treatment with rapamycin alone, did not prevent the production of anti-rat antibodies. The increased production of anti-rat antibody in recipients with rejected grafts and the corresponding lack of antibody production in mice with long-term graft survival, suggests a key mechanism of antibody mediated graft rejection in this model. Indeed, other studies have also found that co-stimulation blockade modulates the antibody response and helps maintain graft function (32).

Neonatal porcine islets have been described as suitable source of islets for transplantation clinically (23;33), and at present represent one the most promising sources of islets to meet the high demand for pancreatic tissue in human islet transplantation. However, many experimental models which are used, implement porcine islets which are transplanted into mice recipients, representing a discordant relationship (27). If porcine islets are to become a source of pancreatic tissue in human islet transplantation where the relation is not as phylogenetically disparate, it is important to compare the mechanism of graft rejection as the relationship between the donor and recipient goes from discordant to concordant or vice versa. Therefore, we performed the present study which represents a concordant xenogeneic islet transplantation, to compare the response of the immune system with that of a discordant islet transplantation which has been previously reported by our lab (20). Previous studies have demonstrated the importance of $CD4^+$ T cells in discordant xenograft rejection, with an emphasis on the indirect pathway of antigen presentation (29;34). However, it has been reported that as the relation between donor and recipient becomes more concordant, that the direct pathway of antigen presentation is increasingly active in graft rejection (35), although the indirect pathway appears to maintain a significant role in graft rejection (36). Our therapy was aimed at preventing both the indirect and direct pathways. Anti-LFA-1 treatment, which primarily targets recipient T and B cells, also has been shown to protect against graft rejection in an indirect antigen presentation dominated model and functions primarily through interrupting T and B cell homing and activation (19-21). The inhibition of anti-rat antibody production in recipient mice treated with anti-LFA-1 suggests that the anti-LFA-1 antibody successfully interrupts the indirect pathway of antigen presentation. This is

substantiated by the fact that T cell dependent antibody responses to xenografts are inferred evidence of indirect antigen recognition since T-B cell activation requires the assistance of helper T cells, with antigens acquired and presented by the specific B cell recognizing the rat antigens. Rapamycin, being a general immunosuppressant, is effective at inhibiting T_H cell proliferation and consequently, reducing the activation of various effector populations including T_C, B cells, NK cells, and macrophages, which can potentially contribute to graft rejection (6;8;9). Additionally, because rapamycin acts as a general immunosuppressant, following treatment, both host and donor immune cells are inhibited. This has immediate implications for both the indirect and the direct pathways. Because the lifetime of naïve passenger leukocytes is limited, treatment with rapamycin for two weeks effectively suppresses the direct pathway, to some extent, in our model.

Our study demonstrated the efficacy of rapamycin and anti-LFA-1 in promoting the survival of rat islet xenografts in BALB/c mice. The importance of LFA-1 in suppressing xenograft rejection in our model was apparent. The combination of rapamycin with anti-LFA-1 was associated with a decrease in the cellular infiltration of the islet grafts as well as a reduction in the production of anti-rat antibody in recipient mice. The results of our study indicate that the combination of short-term immunosuppression with immunomodulation through monoclonal antibody therapy, could form a significant component of future therapeutic standards in clinical islet xenotransplantation.

2.5 REFERENCES

1. Ryan,E.A., Paty,B.W., Senior,P.A., Bigam,D., Alfadhli,E., Kneteman,N.M., Lakey,J.R., and Shapiro,A.M. 2005. Five-year follow-up after clinical islet transplantation. *Diabetes* 54:2060-2069.

2. Almond,P.S., Moss,A., Nakhleh,R.E., Melin,M., Chen,S., Salazar,A., Shirabe,K., and Matas,A.J. 1993. Rapamycin: immunosuppression, hyporesponsiveness, and side effects in a porcine renal allograft model. *Transplantation* 56:275-281.

3. Kneteman,N.M., Cattral,M.C., Warnock,G.L., Scharp,D.W., Jaspan,J.L., and Rajotte,R.V. 1990. Immunosuppression and pancreatic islet transplantation. *Horm.Metab Res.Suppl* 25:193-199.

4. Ryan,E.A., Paty,B.W., Senior,P.A., and Shapiro,A.M. 2004. Risks and side effects of islet transplantation. *Curr.Diab.Rep.* 4:304-309.

5. Shapiro,A.M., Lakey,J.R., Ryan,E.A., Korbutt,G.S., Toth,E., Warnock,G.L., Kneteman,N.M., and Rajotte,R.V. 2000. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N.Engl.J.Med.* 343:230-238.

6. Asnaghi,L., Bruno,P., Priulla,M., and Nicolin,A. 2004. mTOR: a protein kinase switching between life and death. *Pharmacol.Res.* 50:545-549.

7. Inoki,K., Ouyang,H., Li,Y., and Guan,K.L. 2005. Signaling by target of rapamycin proteins in cell growth control. *Microbiol.Mol.Biol.Rev.* 69:79-100.

8. Kwon,G., Marshall,C.A., Pappan,K.L., Remedi,M.S., and McDaniel,M.L. 2004. Signaling elements involved in the metabolic regulation of mTOR by nutrients, incretins, and growth factors in islets. *Diabetes* 53 Suppl 3:S225-S232.

9. Rowinsky,E.K. 2004. Targeting the molecular target of rapamycin (mTOR). *Curr.Opin.Oncol.* 16:564-575.

10. Appel,M.C., Banuelos,S.J., Greiner,D.L., Shultz,L.D., Mordes,J.P., and Rossini,A.A. 2004. Prolonged survival of neonatal porcine islet xenografts in mice treated with a donor-specific transfusion and anti-CD154 antibody. *Transplantation* 77:1341-1349.

11. Garrett,H.E., Jr., Duvall-Seaman,D., Helsley,B., and Groshart,K. 2005. Treatment of vascular rejection with rituximab in cardiac transplantation. *J.Heart Lung Transplant.* 24:1337-1342.

12. Rosenberg,P.B., Vriesendorp,A.E., Drazner,M.H., Dries,D.L., Kaiser,P.A., Hynan,L.S., Dimaio,J.M., Meyer,D., Ring,W.S., and Yancy,C.W. 2005. Induction therapy with basiliximab allows delayed initiation of cyclosporine and preserves

renal function after cardiac transplantation. *J.Heart Lung Transplant.* 24:1327-1331.

13. Rossi,J.F., Fegueux,N., Lu,Z.Y., Legouffe,E., Exbrayat,C., Bozonnat,M.C., Navarro,R., Lopez,E., Quittet,P., Daures,J.P. *et al.* 2005. Optimizing the use of anti-interleukin-6 monoclonal antibody with dexamethasone and 140 mg/m2 of melphalan in multiple myeloma: results of a pilot study including biological aspects. *Bone Marrow Transplant.* 36:771-779.

14. Schroeder,G., Risch,K., Kotsch,K., Siepert,A., Brock,J., Nickel,P., Reinke,P., Ritter,T., Volk,H.D., and Lehmann,M. 2004. FTY720 prevents anti-CD4 mAb-induced tolerance but cannot reverse established tolerance in a rat kidney transplantation model. *Am.J.Transplant.* 4:863-871.

15. Watson,C.J., Bradley,J.A., Friend,P.J., Firth,J., Taylor,C.J., Bradley,J.R., Smith,K.G., Thiru,S., Jamieson,N.V., Hale,G. *et al.* 2005. Alemtuzumab (CAMPATH 1H) induction therapy in cadaveric kidney transplantation--efficacy and safety at five years. *Am.J.Transplant.* 5:1347-1353.

16. Arai,K., Sunamura,M., Wada,Y., Takahashi,M., Kobari,M., Kato,K., Yagita,H., Okumura,K., and Matsuno,S. 1999. Preventing effect of anti-ICAM-1 and anti-LFA-1 monoclonal antibodies on murine islet allograft rejection. *Int.J.Pancreatol.* 26:23-31.

17. Fabien,N., Bergerot,I., Orgiazzi,J., and Thivolet,C. 1996. Lymphocyte function associated antigen-1, integrin alpha 4, and L-selectin mediate T-cell homing to the pancreas in the model of adoptive transfer of diabetes in NOD mice. *Diabetes* 45:1181-1186.

18. Nicolls,M.R., Coulombe,M., Yang,H., Bolwerk,A., and Gill,R.G. 2000. Anti-LFA-1 therapy induces long-term islet allograft acceptance in the absence of IFN-gamma or IL-4. *J.Immunol.* 164:3627-3634.

19. Nicolls,M.R., Coulombe,M., Beilke,J., Gelhaus,H.C., and Gill,R.G. 2002. CD4-dependent generation of dominant transplantation tolerance induced by simultaneous perturbation of CD154 and LFA-1 pathways. *J.Immunol.* 169:4831-4839.

20. Rayat,G.R. and Gill,R.G. 2005. Indefinite survival of neonatal porcine islet xenografts by simultaneous targeting of LFA-1 and CD154 or CD45RB. *Diabetes* 54:443-451.

21. Schneider,H., Valk,E., da Rocha,D.S., Wei,B., and Rudd,C.E. 2005. CTLA-4 up-regulation of lymphocyte function-associated antigen 1 adhesion and clustering as an alternate basis for coreceptor function. *Proc.Natl.Acad.Sci.U.S.A* 102:12861-12866.

22. Lebwohl,M., Tyring,S.K., Hamilton,T.K., Toth,D., Glazer,S., Tawfik,N.H., Walicke,P., Dummer,W., Wang,X., Garovoy,M.R. *et al.* 2003. A novel targeted T-cell modulator, efalizumab, for plaque psoriasis. *N.Engl.J.Med.* 349:2004-2013.

23. Korbutt,G.S., Elliott,J.F., Ao,Z., Smith,D.K., Warnock,G.L., and Rajotte,R.V. 1996. Large scale isolation, growth, and function of porcine neonatal islet cells. *J.Clin.Invest* 97:2119-2129.

24. Koulmanda,M., Qipo,A., Smith,R.N., and Auchincloss,H., Jr. 2003. Pig islet xenografts are resistant to autoimmune destruction by non-obese diabetic recipients after anti-CD4 treatment. *Xenotransplantation.* 10:178-184.

25. MacKenzie,D.A., Hullett,D.A., and Sollinger,H.W. 2003. Xenogeneic transplantation of porcine islets: an overview. *Transplantation* 76:887-891.

26. Tang,T.H., Li,C.L., Li,X., Jiang,F.Q., Zhang,Y.K., Ren,H.Q., Su,S.S., and Jiang,G.S. 2004. Immune tolerance in pancreatic islet xenotransplantation. *World J.Gastroenterol.* 10:1457-1461.

27. Takahashi,K., Rooney,A.P., and Nei,M. 2000. Origins and divergence times of mammalian class II MHC gene clusters. *J.Hered.* 91:198-204.

28. Koulmanda,M., Laufer,T.M., Auchincloss,H., Jr., and Smith,R.N. 2004. Prolonged survival of fetal pig islet xenografts in mice lacking the capacity for an indirect response. *Xenotransplantation.* 11:525-530.

29. Rayat,G.R., Johnson,Z.A., Beilke,J.N., Korbutt,G.S., Rajotte,R.V., and Gill,R.G. 2003. The degree of phylogenetic disparity of islet grafts dictates the reliance on indirect CD4 T-cell antigen recognition for rejection. *Diabetes* 52:1433-1440.

30. Korbutt,G.S., Elliott,J.F., Ao,Z., Smith,D.K., Warnock,G.L., and Rajotte,R.V. 1996. Large scale isolation, growth, and function of porcine neonatal islet cells. *J.Clin.Invest* 97:2119-2129.

31. Larsson,L.C., Corbascio,M., Widner,H., Pearson,T.C., Larsen,C.P., and Ekberg,H. 2002. Simultaneous inhibition of B7 and LFA-1 signaling prevents rejection of discordant neural xenografts in mice lacking CD40L. *Xenotransplantation.* 9:68-76.

32. Wu,G., Pfeiffer,S., Schroder,C., Zhang,T., Nguyen,B.N., Lea,W., Kelishadi,S., Atkinson,J.B., Schuurman,H.J., White,D.J. *et al.* 2005. Co-stimulation blockade targeting CD154 and CD28/B7 modulates the induced antibody response after a pig-to-baboon cardiac xenograft. *Xenotransplantation.* 12:197-208.

33. Kin,T., Korbutt,G.S., Kobayashi,T., Dufour,J.M., and Rajotte,R.V. 2005. Reversal of diabetes in pancreatectomized pigs after transplantation of neonatal porcine islets. *Diabetes* 54:1032-1039.

34. Olack,B.J., Jaramillo,A., Benshoff,N.D., Kaleem,Z., Swanson,C.J., Lowell,J.A., and Mohanakumar,T. 2002. Rejection of porcine islet xenografts mediated by CD4+ T cells activated through the indirect antigen recognition pathway. *Xenotransplantation.* 9:393-401.

35. Andres,A., Toso,C., Morel,P., Bosco,D., Bucher,P., Oberholzer,J., Mathe,Z., Mai,G., Wekerle,T., Berney,T. *et al.* 2005. Macrophage depletion prolongs discordant but not concordant islet xenograft survival. *Transplantation* 79:543-549.

36. Singh,N.P., Guo,L., Que,X., and Shirwan,H. 2004. Blockade of indirect recognition mediated by CD4+ T cells leads to prolonged cardiac xenograft survival. *Xenotransplantation.* 11:33-42.

CHAPTER 3

RAPAMYCIN AND ANTI-LFA-1 TREATMENT RESULTS IN THE INDUCTION OF TOLERANCE TO RAT ISLET XENOGRAFTS

3.1 INTRODUCTION

Islet transplantation has provided an attractive alternative treatment for patients with type 1 diabetes mellitus (T1DM). However, islet transplantation is not available to the vast majority of diabetics because of two main factors. The first is the shortage of donor pancreases, which is further compounded by the observation that typically more than one donor pancreas is required to achieve insulin independence (1;2). The second, is need for continuous immunosuppression, which is associated with a number of harmful side effects (3;4). These shortcomings have prompted investigators to seek out new sources of insulin producing tissue and new methods of preventing the rejection of the transplanted islets, which are not associated with the toxicity of the currently implemented immunosuppressive regimen. The focus of our research is to develop a tolerizing regimen, which is given short-term, and is capable of sustaining long-term acceptance of transplanted islet grafts, thereby circumventing the need for continuous immunosuppression. Xenogeneic sources of islets could provide a potentially unlimited supply of islets for transplantation in humans. While many xenogeneic sources of insulin producing tissue have been used experimentally, pigs are arguably the most ideal source for humans. Pig islets are often transplanted into mice to investigate effective anti-rejection therapies. This relationship between donor and recipient is termed discordant, due to the phylogenetic disparity between pigs and mice. If porcine islets are to become a

source of islets in clinical transplantation, the immune response must be examined as the relation between donor and recipient becomes more concordant, as pigs are more closely related to humans than pigs are to mice (5). Rat islet donors transplanted into diabetic mice provide an attractive method of studying the immune response and the effectiveness of anti-rejection therapies in concordant xenogeneic transplantation. By elucidating the mechanisms of concordant xenograft rejection, this information can be applied to devising effective anti-rejection therapies for clinical islet transplantation.

Rapamycin (sirolimus) forms a critical component of the immunosuppressive regimen of the Edmonton protocol. Rapamycin is a potent immunosuppressive drug and its effectiveness is attributed to its ability to inhibit T and B cell proliferation by inhibiting the mammalian target of rapamycin (mTOR). Recently, several studies have been published which report that rapamycin contributes to the induction of antigen specific tolerance (6;7) as well as selectively expanding $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells (8), and preventing new onset diabetes and inducing immune tolerance in NOD mice (9).

Leukocyte function associated antigen-1 (LFA-1) has emerged as an important contributor of T cell activation since it was discovered that LFA-1 interaction with ICAM-1 on APCs produces its own positive signal (10). As an integrin, LFA-1 is also involved in the migration of lymphocytes, making LFA-1 an especially attractive target of monoclonal antibodies for the prevention of rejection of transplanted tissue (11). The targeting of recipient LFA-1 and donor ICAM-1 has been shown to prolong concordant islet xenotransplantation (12;13), and in discordant islet transplantation the ICAM-1/LFA-1 interaction was found to be critical for xenograft rejection (14). Recently, a

humanized form of anti-LFA-1, Efalizumab was approved for phase IV clinical trials for the treatment of psoriasis (15).

We have previously reported on the efficacy of a short-term administration of rapamycin with anti-LFA-1 monoclonal antibody in the prevention of concordant islet xenograft rejection in BALB/c mice. In this study, we set out to determine whether the combination of rapamycin and anti-LFA-1 was merely prolonging graft survival or whether this therapy was resulting the induction of tolerance to the rat islet xenografts.

3.2 MATERIALS AND METHODS

3.2.1 EXPERIMENTAL ANIMALS

Inbred male BALB/cByJ (BALB/c, H-2^d), BALB/c rag-/- (C.129S7(B6)-*Rag1*^{tm1Mom}/J) and C57BL/6J (B6, H-2^b) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and Wistar-Furth (WF, $RT1^U$) and Lewis ($RT1^l$) rats were purchased from Harlan Laboratories (Indianapolis, IN). Recipient BALB/c and BALB/c rag -/- mice were rendered diabetic by a single intraperitoneal injection of streptozotocin (250 mg/kg body weight; Sigma-Aldrich, Oakville, ON) 4 to 5 days before transplantation. All diabetic recipients had two consecutive non-fasting blood glucose levels above 17 mmol/l prior to transplantation. The blood glucose of each recipient mouse was monitored via blood samples from the tail vein. Glucose levels were measured using a OneTouch Ultra glucometer (LifeScan, Burnaby, BC). All mice and rats were housed under pathogen-free conditions, fed standard laboratory food and water *ad libitum*, and cared for according to the guidelines established by the Health Sciences Animal Policy and Welfare Committee.

3.2.2 ISLET ISOLATION AND TRANSPLANTATION

WF rat pancreatic islets were isolated via distension of the pancreas, followed by collagenase (Sigma, Oakville, ON) digestion, density gradient centrifugation with dextran (Sigma, Oakville, ON) and handpicking of the islets. Islets were cultured overnight in Hams F10 media at 37°C and 5% CO_2. A total of 500 isolated rat islets were transplanted under the left kidney capsule of diabetic mice as previously described (16). Engraftment was considered successful when the blood glucose level was ≤10 mM. Graft rejection was defined as the first of consecutive days of hyperglycemia (≥2 mM glucose) and rejection was confirmed by histological analysis of the graft. Survival nephrectomy of the graft-bearing kidney was performed on a subset of recipients with long-term graft function (>100 days post-transplant) for the confirmation of graft-dependent euglycemia.

3.2.3 ANTI-REJECTION THERAPIES

BALB/c transplant recipients received the following anti-rejection treatment intraperitoneally: Rapamycin (0.2 mg/kg on days 0 thru 14 post-transplantation; a generous gift from Dr. James Shapiro, Edmonton, AB) and anti-LFA-1 monoclonal antibody (KBA; rat IgG2a; hybridoma kindly provided by Dr. Ron Gill, Denver CO; 200μg on days 0, 1, 7, 14, 21 and 28 post-transplant) which is also referred to as the combination therapy.

3.2.4 PREPARATION AND ADOPTIVE TRANSFER OF DONOR SPLEEN CELLS

Spleens were isolated from naïve BALB/c mice and BALB/c mice that were transplanted and treated with rapamycin and anti-LFA-1, and maintained normoglycemia

for greater than 100 days post-transplantation (referred to as tolerant spleen cells). The spleens were transferred aseptically to a petri dish containing cold sterile saline. The spleens were mechanically disrupted by grinding the spleen between two sterile slides. The cell suspension was collected and counted via hematocytometer and light microscopy excluding erythrocytes in the count. A $50x10^6$ cell aliquot was adoptively transferred intraperitoneally via 25G needle. Spleen cells were adoptively transferred to BALB/c rag -/- mice which lack T and B lymphocytes. These mice were previously rendered diabetic by STZ injection and transplanted with either Wistar-Furth or Lewis rat islets as described previously. No anti-rejection therapy was given to the BALB/c rag-/- mice and the islet grafts were allowed to establish for 100 days prior to adoptive transfer of spleen cells. Mice that were adoptively transferred with donor spleen cells, were monitored as described previously for a period of 50 days post-adoptive transfer.

3.2.5 IMMUNOHISTOCHEMICAL STAINING

Four μm-thick sections of formalin-fixed tissues were stained to determine the presence of insulin containing cells. Insulin containing cells were detected by applying guinea-pig anti-porcine insulin primary antibody (1:1000; DAKO Corporation, Carprinteria, CA) for 30 min, followed by the addition of biotinylated goat anti-guinea pig IgG secondary antibody (1:200; Vector Laboratories, Burlington, ON). The avidin-biotin complex/horseradish peroxidase (ABC/HP; Vector Laboratories) and 3, 3-diaminobenzidinetetrahydrochloride (DAB; BioGenex, San Ramon, CA) was used to produce a brown color. All sections were counterstained with Harris' hematoxylin and eosin.

Subpopulations of T cells (CD4 and CD8), granulocytes, macrophages and natural killer cells (CD11b) were also determined on tissues immersed in OCT compound and snap frozen at –80° C. Five μm sections of tissue were air dried for 10 min then fixed in acetone for 3 min at 4°C. These sections were then washed in PBS and non-specific binding was eliminated by incubating the tissue section in 2% FBS/PBS for 20 min. In addition, endogenous biotin or biotin-binding proteins present in the section were also eliminated using the avidin/biotin blocking kit (Vector Laboratories, Inc., Burlington, ON). Rat anti-mouse CD4 antibody (1:100; BD Pharmingen) or rat anti-mouse CD8 antibody (1:100; BD Pharmingen, Mississauga, ON) or rat anti-mouse CD11b (1:500; BD Pharmingen, Mississauga, ON) was applied to each section for 30 min at room temperature. Biotinylated rabbit anti-rat IgG secondary antibody (1:200; Vector Laboratories, Inc. Burlington, ON) was added and incubated for 20 min. Avidin-Biotin Complex/Horseradish Peroxidase (ABC/HP) Reagent (Vector Laboratories, Inc. Burlington, ON) was applied and 3, 3-diaminobenzidinetetrahydrochloride (DAB; BioGenex, San Ramon, CA) was used to produce a brown color. Sections were counterstained with Harris' hematoxylin.

3.2.6 SKIN GRAFTING

Skin grafts were harvested from C57BL/6 mice, which acted as a 3rd party graft. Syngeneic BALB/c skin graft served as a control. Briefly, the dorsum of the mouse was shaved and sterilized using 95% ethanol. Using a 8mm punch biopsy (Acuderm, Fort Lauderdale, FL), a circular piece of full thickness skin was isolated and rinsed in saline followed by a penicillin/streptomycin solution followed by another wash in sterile saline.

The skin was then placed on the recipient's 6mm wound and sutured in place. The skin was covered with sterile gauze soaked in saline and wrapped using an adhesive bandage. Dressing changes were performed 2 days following skin transplantation and every week thereafter.

3.2.7 SERUM ANTIBODY MEASUREMENT

We determined the production of mouse anti-rat antibodies in BALB/c rag -/- mice adoptively transferred with naïve, tolerant or tolerant mixed with naïve spleen cells. The levels of mouse anti-rat IgG antibodies were measured from the blood serum of each adoptively transferred mouse using flow cytometry. Peripheral blood samples from mice were collected at the time of rejection or following long-term islet xenograft survival. The serum was isolated via incubation at room temperature for at least 3 hours, followed by centrifugation at 10,000 rpm for 10 min. Rat spleen cells ($1x10^6$) obtained from the same rat islet donors were incubated with mouse serum at 1/128 dilution for 1 hour at 37°C (5% CO_2, 95% air). Spleen cells were then washed with PBS and incubated with FITC-conjugated goat anti-mouse IgG (rat adsorbed, 1:100; Southern Biotechnology Associates, Birmingham, AL) for 1 hour at 4°C. The percentage of cells bound to antibody was detected from single-parameter fluorescence histograms on a BD FACSCalibur (BD Bioscience, ON) after gating on viable mononuclear cells. Controls for this experiment include sera from non-transplanted naïve BALB/c rag -/- mice, unstained rat spleen cells, and rat spleen cells stained with secondary antibody alone without mouse serum.

3.2.8 STATISTICAL ANALYSIS

Statistical differences in graft survival among groups were analyzed using the Kaplan-Meier log rank test. A p value of less than 0.05 was considered to be statistically significant.

3.3 RESULTS

3.3.1 COMBINATION OF RAPAMYCIN AND ANTI-LFA-1 INDUCES INDEFINITE RAT ISLET XENOGRAFT SURVIVAL

We have previously reported that treatment with rapamycin and anti-LFA-1 monoclonal antibody prevents the rejection of rat islet xenografts in BALB/c mice. In an effort to determine whether this combination therapy was merely delaying graft rejection or was inducing long-term tolerance to the islet xenografts, we allowed a subset of mice to go past 100 days post-transplantation with no additional anti-rejection therapy. All five mice that were studied past 100 days maintained normoglycemia for greater than 200 post-transplantation (Figure 3-1). A nephrectomy of the islet graft bearing kidney was performed in all mice to verify the presence of islet graft dependent euglycemia. Following the removal of the islet graft bearing kidney, all five mice returned to hyperglycemia. Histological analysis of the islet grafts revealed robust insulin positive cells in most grafts and a moderate level of islet graft infiltration including some sporadic $CD4^+$ T cells and virtually no $CD8^+$ T cells (Figure 3-2).

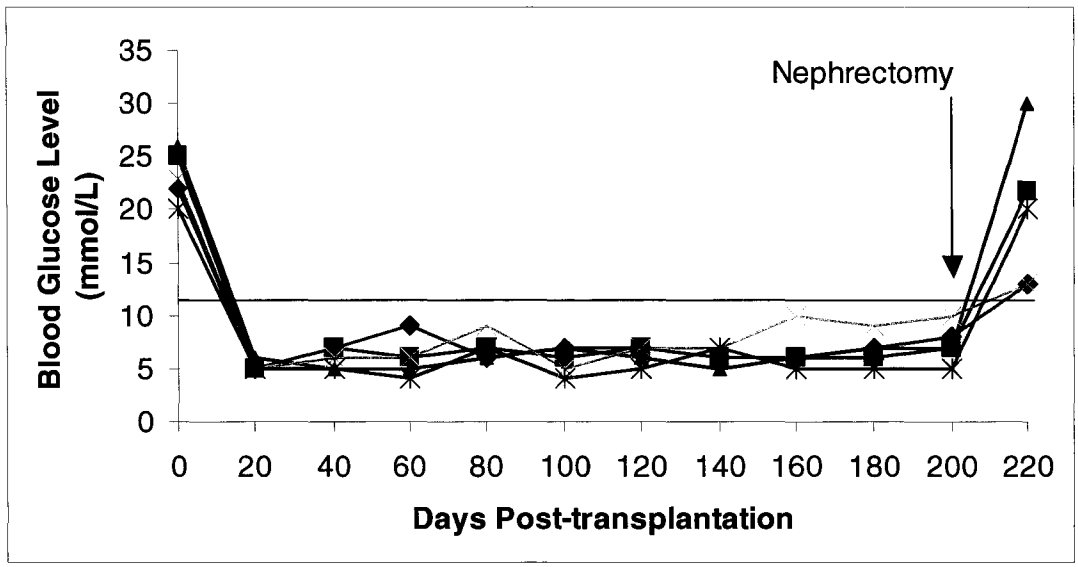


Figure 3-1. Blood glucose levels of five mice receiving a short-term therapy of rapamycin and anti-LFA-1 and monitored for 200 days post-transplantation. A survival nephrectomy was performed at day 200 to verify the presence of graft dependent euglycemia. All mice following the removal of the islet graft bearing kidney reverted to hyperglycemia.

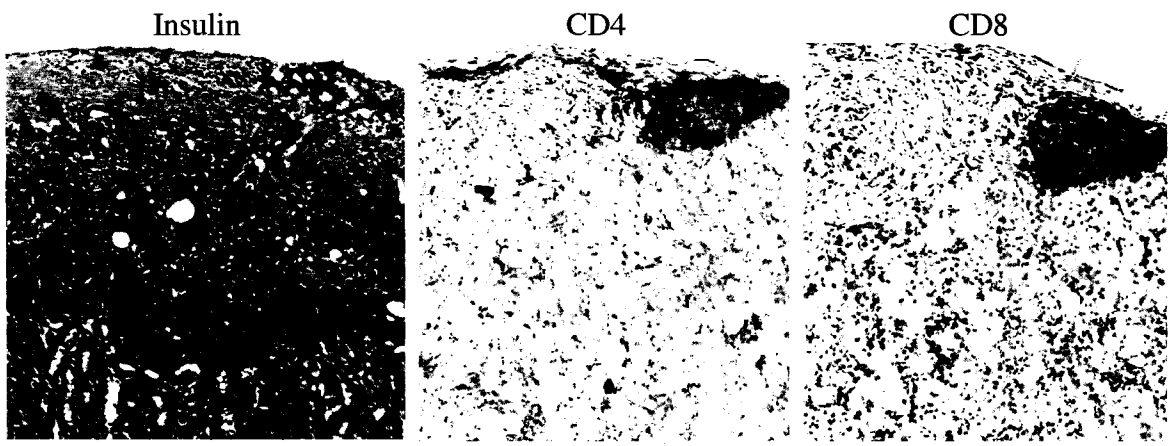


Figure 3-2. Immunohistochemical analysis of islet grafts for insulin staining and characterization of infiltrating mononuclear cell types at 200 days post-transplantation. Staining for CD4 ($CD4^+$ T cells), and CD8 ($CD8^+$ T cells) was performed to identify these populations of cells within the islet grafts. Positive cells are indicated by brown staining. (Magnification 100x)

3.3.2 ADOPTIVE TRANSFER OF TOLERANT SPLEEN CELLS RESULTS IN DELAYED REJECTION

Spleen cells of naïve or tolerant mice were adoptively transferred to BALB/c rag -/- mice. Following a post-transplantation period of 100 days to allow WF rat islet engraftment, $50x10^6$ naïve spleen cells were adoptively transferred to BALB/c rag-/- mice. All mice (3/3) rejected their grafts acutely with a mean survival time (MST) of 13.7 days following adoptive transfer (Table 3-1). The majority of mice that were adoptively transferred with tolerant spleen cells rejected their WF rat islet grafts (7/8). However, mice that were transferred with tolerant spleen cells demonstrated a delay in the rejection of the islet xenografts (MST of 23.8 days) compared to mice receiving naïve spleen cells ($p = 0.24$ vs naïve). In addition, one mouse receiving tolerant spleen cells maintained normoglycemia for 50 days post-adoptive transfer, which was the end point of the study. Spleen cells from naïve mice and tolerant mice were combined in a 1:1 ratio and adoptively transferred to BALB/c rag-/- mice to examine the effect of tolerant spleen cells on the naïve spleen cells in WF rat islet graft rejection. One mouse maintained normoglycemia for greater than 50 days (1/6). The MST of mice transferred with a combination of naïve and tolerant spleen cells was 22.5 days, which was comparable to mice receiving tolerant spleen cells (MST of 23.8 days) alone (Table 3-1) ($p = 0.66$ vs naïve). In a similar experiment, BALB/c rag -/- mice were transplanted as before, but instead received either a Lewis rat islet xenograft or a C57BL/6 islet allograft. Following adoptive transfer with tolerant spleen cells, all mice (6/6) rejected their Lewis rat islet xenografts with a MST of 26.7 days. BALB/c rag -/- mice transplanted with C57BL/6

islets and reconstituted with $50x10^6$ tolerant spleen cells, all rejected their islet grafts acutely (3/3) with a MST of 12.3 days post-adoptive transfer (Table 3-2).

Adoptive Transfer Spleen Cell Donor	n	Graft Survival (Days)	MST (Days) ± SD
Naive spleen cells ($50x10^6$ cells)	3	10, 14, 17	13.7 ± 3.5
Tolerant ($50x10^6$ cells) spleen cells	8	9, 13, 14, 14, 20, 30, 40, >50	23.8 ± 14.8
Naive/Tolerant ($50x10^6$ each) spleen cells	6	7, 11, 12, 14, 41, >50	22.5 ± 18.2

Table 3-1. Graft survival in BALB/c rag -/- mice adoptively transferred with spleen cells from either naïve mice, tolerant mice, or a combination of the two types of donors. Mice receiving naïve spleen cells acutely rejected their islet grafts. Mice receiving spleen cells from tolerant mice, or a combination of naïve and tolerant spleen cells, demonstrated a delay in rejection as measured by blood glucose, with one mouse from each group maintaining its Wistar-Furth rat islet xenograft for over 50 days. Mean graft survival time (MST) is reported in days following adoptive transfer ± standard deviation.

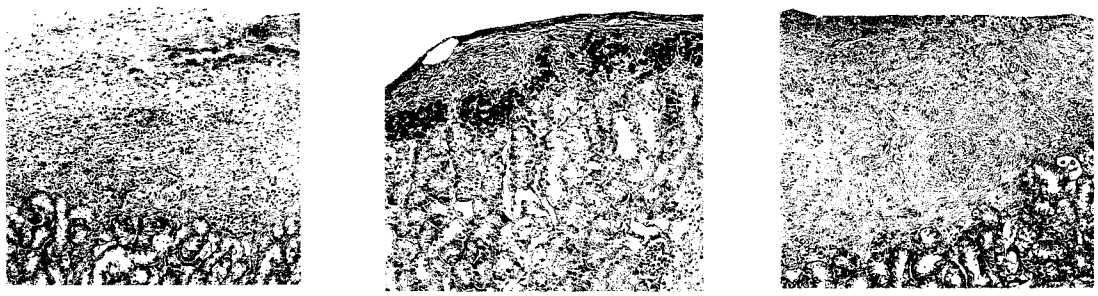
Islet Donor	n	Graft Survival (Days)	MST (Days) ± SD
Lewis rat	6	10, 13, 19, 34, 41, 43	26.7 ± 14.5
C57BL/6 mice	3	9, 9, 19	12.3 ± 5.8

Table 3-2. Graft survival in BALB/c rag -/- mice transplanted with either xenogeneic Lewis rat islets or allogeneic C57BL/6 mouse islets and adoptively transferred with tolerant spleen cells. Mean graft survival time (MST) is reported in days following adoptive transfer ± standard deviation.

3.3.3 ISLET GRAFT REJECTION IS ASSOCIATED WITH GRAFT INFILTRATION BY MONONUCLEAR CELLS

All grafts were collected either following rejection or after 50 days post-adoptive transfer, for histologic examination of the islet graft and identification of cellular infiltrate. Grafts of BALB/c rag-/- mice adoptively transferred with naïve spleen cells lacked insulin positive staining and a large influx of mononuclear cells was present at the

site of the graft (Figure 3-3). Intense staining was detected for $CD4^+$ T cells and in addition, $CD8^+$ T cells and $CD11b^+$ cells could be detected at the site of the graft albeit to a lesser extent than $CD4^+$ T cells (Figure 3-4). The majority of mice that were adoptively transferred with naïve and tolerant spleen cells combined, lacked insulin positive staining. In addition, a heavy infiltration of $CD4^+$ T cells could be detected at the site of the graft. $CD8^+$ T cells and $CD11b^+$ cells were also detected, but again, were found at decreased intensity when compared to amount of $CD4^+$ T cell staining. Insulin positive staining was scarce or not detected in the majority of mice receiving tolerant spleen cells (data not shown), however a significant amount of insulin positive staining was detected in the mouse with >50 days graft survival following adoptive transfer (Figure 3-3). In addition, the intensity of staining for CD4, CD8 and CD11b, was diminished in mice receiving tolerant spleen cells, although at least some $CD4^+$, $CD8^+$, and $CD11b^+$ cells could be detected at the site of the xenograft (Figure 3-4).



Adoptively Transferred with Naïve Spleen Cells

Adoptively Transferred with Tolerant Spleen Cells

Adoptively Transferred with Naïve/Tolerant Spleen Cells

Figure 3-3. Histologic examination of islet xenografts at the time of rejection or 50 days post-adoptive transfer. Islet grafts of BALB/c rag -/- mice adoptively transferred with naïve spleen cells demonstrate an intense infiltration of mononuclear cells and no insulin positive cells (brown color). Islet grafts of one BALB/c rag -/- mice adoptively transferred with tolerant spleen cells was positive for insulin staining. No insulin positive staining was detected in the grafts of mice adoptively transferred with a mixture of naïve and tolerant spleen cells. (Magnification 100x)

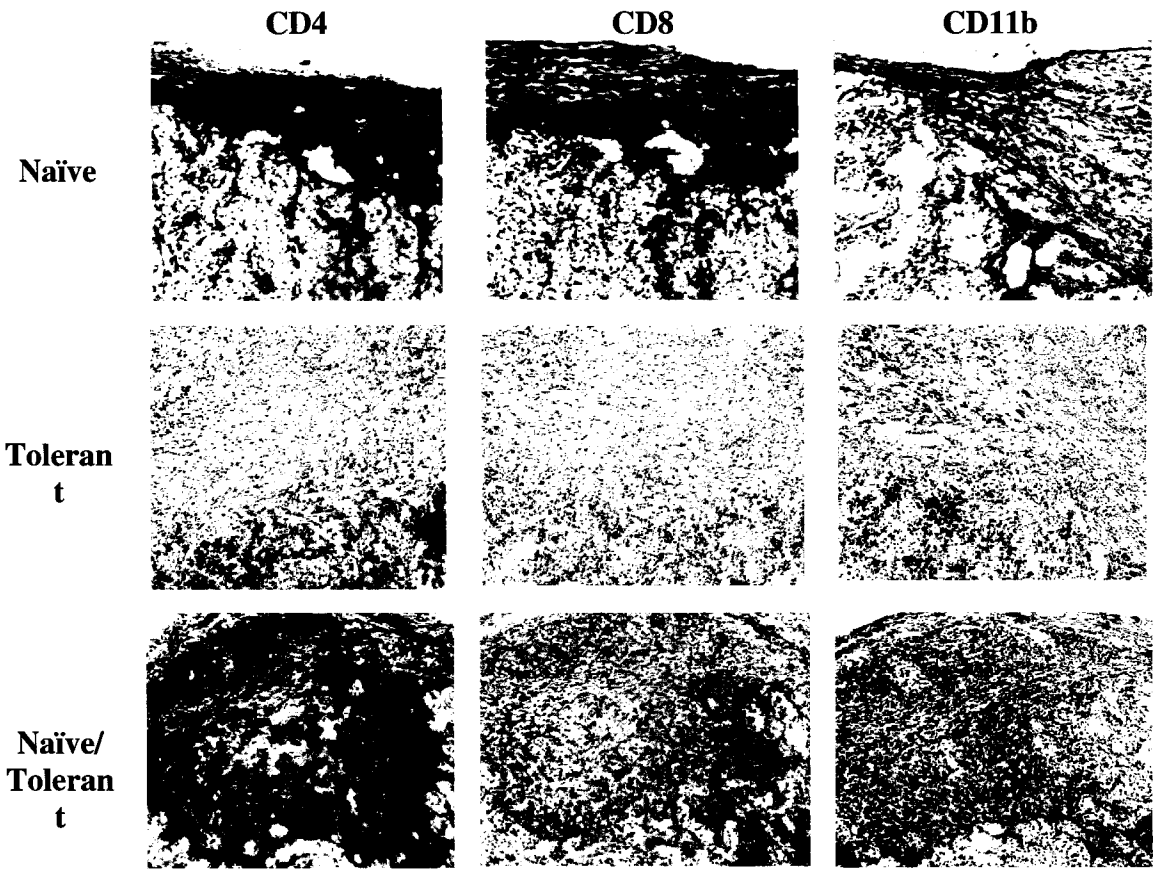


Figure 3-4. Immunohistochemical analysis of islet grafts for characterization of infiltrating mononuclear cell types. Staining for CD4 ($CD4^+$ T cells), CD8 ($CD8^+$ T cells) and CD11b (monocytes/macrophages, NK and granulocytes) was performed to identify these populations of cells within the islet grafts. Positive cells are indicated by brown staining. (Magnification 100x)

3.3.4 ISLET GRAFT REJECTION IS ASSOCIATED WITH AN INCREASE IN MOUSE ANTI-RAT ANTIBODY PRODUCTION

Adoptively transferred recipient's sera was analyzed using flow cytometry. In mice that received naïve spleen cells, and subsequently rejected their WF rat xenografts, there was a significant shift of the histogram peak compared to the negative controls, indicating that a significant production of mouse anti-rat antibody had occurred (Figure

3-5). In mice that were adoptively transferred with naïve and tolerant spleen cells combined, a significant level of anti-rat antibody was detected. Mice that received tolerant spleen cells and rejected their islet grafts, had increased levels of anti-rat antibodies present in their sera compared to untransplanted controls. Anti-rat antibody production was closely correlated to graft rejection. One BALB/c rag-/- mouse receiving tolerant spleen cells, that maintained xenograft function for greater than 50 days, had no detectable increase in mouse anti-rat antibody production compared to untransplanted controls (Figure 3-5).

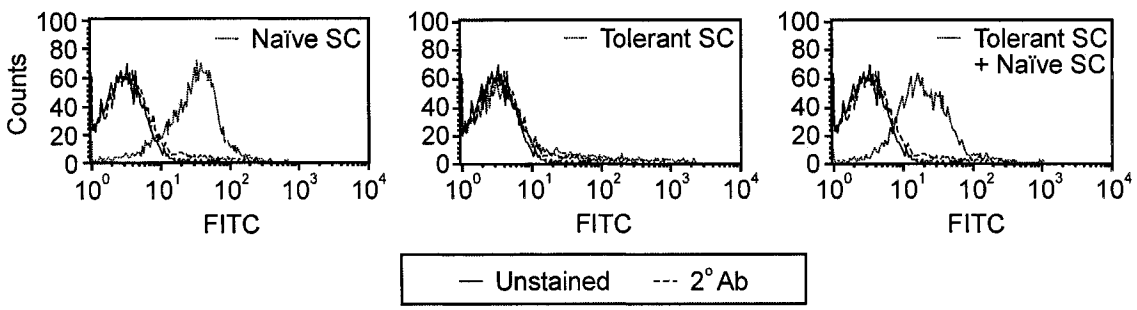


Figure 3-5. Analysis of mouse anti-rat antibodies in BALB rag -/- mice using flow cytometry. An increase in the level of mouse anti-rat antibodies was observed in the serum of mice adoptively transferred with spleen cells from naïve and naïve plus tolerant mice. No increase in anti-rat antibodies could be detected in the serum of the mouse with graft function after 50 days following adoptive transfer of tolerant spleen cells. The production of mouse anti-rat antibodies was closely coupled to islet graft rejection events.

3.3.5 RAPAMYCIN AND ANTI-LFA-1 TREATED RECIPIENTS ARE TOLERANT OF A SECOND IDENTICAL RAT ISLET XENOGRAFT

BALB/c mice were transplanted with WF rat islets and treated with rapamycin and anti-LFA-1 as before. Following at least 100 days of normoglycemia, a survival nephrectomy was performed of the islet graft bearing kidney. Graft dependent

euglycemia was confirmed, as all mice reverted to hyperglycemia. Once two hyperglycemic readings were obtained, the contralateral kidney (right) was transplanted either with the same WF rat strain or a Lewis rat islet graft. No anti-rejection therapy was administered whatsoever. Of mice that were transplanted with WF rat islets for their second islet transplant, 4 of 4 maintained normoglycemia for greater than 100 days post-transplant, with no requirement for anti-rejection therapy (Table 3-3). To verify that these mice were still capable of mounting an appropriate immune response, third party skin grafts were performed. While a syngeneic BALB/c mouse skin graft was accepted, C57BL/6 mouse skin grafts were ultimately rejected (data not shown). A similar experiment was performed but instead of a re-transplantation with a WF rat graft as before, mice received a Lewis rat islet graft. Interestingly, mice that received a second islet transplant of Lewis rat islets, 2 of 3 mice rejected their islet grafts (as of the time of writing)(Table 3-3).

Islet Donor	n	Graft Survival (Days)
Wistar-Furth rat	5	>88*, >100, >100, >100, >100,
Lewis rat	3	56, 63, >87*

Table 3-3. Graft survival measured in BALB/c mice following a second islet transplant of the right kidney capsule. BALB/c mice were either transplanted with the identical donor as the first transplant (Wistar-Furth rat) or with a different strain of islet donor (Lewis rat). No anti-rejection therapy was administered. * indicates that this mouse is still ongoing at the time of writing.

3.4 DISCUSSION

The aim of this study was to determine whether the combination of rapamycin and anti-LFA-1 was merely prolonging graft survival or whether this therapy was mediating the induction of tolerance to the rat islet xenografts. Rapamycin's beneficial effect in

preventing the rejection of allo-islet grafts has been clearly demonstrated clinically (17). Historically however, immunosuppressive drugs including rapamycin, have largely been classified as tolerance "unfriendly". Recently however, an abundance of literature has uncovered that the stigma of immunosuppressive drugs acting as a barrier to tolerance, may not apply to rapamycin (6-9). Therefore, we tested the efficacy of rapamycin along with another effective anti-rejection agent, anti-LFA-1, to induce tolerance to concordant islet xenografts in BALB/c mice.

To determine whether the combination of this anti-rejection therapy was merely delaying graft rejection or was in fact inducing tolerance to the rat islet xenografts, we monitored a subset of mice past 100 days to determine whether the mice would eventually reject their WF rat islet graft. Of the mice that were monitored, 5/5 maintained normoglycemia for greater than 200 days post-transplantation or 172 days after the last anti-rejection therapy was administered. This suggests that rapamycin and anti-LFA-1 is extremely efficacious at inducing indefinite concordant islet xenograft survival.

To test whether tolerance was transferable from mice receiving rapamycin and anti-LFA-1 to mice with an identical WF rat islet graft, the spleen cells of combination treated mice were isolated and adoptively transferred to BALB/c rag-/- mice who were transplanted with an identical WF rat islet graft. Because BALB/c rag-/- mice lack T and B cells, this model allowed us to determine whether the spleen cells being transferred were tolerant of the WF rat islet graft or whether they would reject the graft, without the interference of the host's adaptive immune system. Spleen cells from naïve untransplanted BALB/c mice were capable of acutely rejecting WF rat islet grafts in

BALB/c rag-/- mice. Despite a lack of rejection in BALB/c mice following the combination anti-rejection therapy, spleen cells from these mice were capable of rejecting an identical WF rat islet graft when adoptively transferred to BALB/c rag-/- mice, in the majority of cases, albeit at a slower rate compared to mice which received naïve spleen cells. To determine if tolerance is induced dominantly, spleen cells from tolerant mice were combined with spleen cells from naïve mice to test whether the tolerant cells were capable of suppressing the naïve spleen cells. While the kinetics of rejection were delayed when the tolerant cells were added to the naïve cells, the majority of mice receiving tolerant and naïve cells eventually rejected their rat islet xenografts. To investigate whether tolerant spleen cells were strain or species specific, islet grafts from Lewis rats or C57BL/6 mice were transplanted into BALB/c rag-/- mice. Following a 100 day period to allow the islets to engraft, BALB/c rag-/- mice were reconstituted with $50x10^6$ tolerant spleen cells and their blood glucose levels were measured. BALB/c rag-/- mice that were transplanted with Lewis rat islets, all rejected their islet grafts (n=6) after transfer of tolerant spleen cells. Likewise, BALB/c rag-/- mice transplanted with C57BL/6 islet grafts, all rejected their islet grafts upon transfer of tolerant spleen cells with similar kinetics as mice receiving naïve spleen cells alone. This evidence would suggest that spleen cells from rapamycin and anti-LFA-1 treated mice, induce incomplete tolerance once adoptively transferred. This is based on the observation that 3 of the 8 mice receiving tolerant spleen cells rejected their islet grafts slower compared to those mice receiving naïve spleen cells alone, with one mouse maintaining islet graft function for greater than 50 days post-transfer. It appears that the tolerant spleen cells were capable of suppressing the acute rejection caused by naïve spleen cells when the two

populations were mixed and transferred, as the MST of mice was similar to those mice which received tolerant cells only (22.5 vs 23.8 days respectively). However, the majority of mice receiving naïve and tolerant spleen cells rejected their grafts, emphasizing that the spleen cells from mice treated with rapamycin and anti-LFA-1 are incompletely tolerant. While tolerant spleen cells were capable of rejecting Lewis rat islet grafts with similar efficiency as WF rat islets (26.7 vs 23.8 days respectively), when the tolerant cells were transferred to BALB/c rag-/- mice with a C57BL/6 islet graft, the graft was rejected with similar kinetics as those which were exposed to naïve spleen cells (12.3 vs 13.7 days respectively). This would suggest that while tolerance is incomplete in this model, it appears to be species specific, however further investigation is required to fully substantiate this conclusion. Although the mechanism behind which the tolerant spleen cells reject islet xenografts at a slower rate has yet to be elucidated, the possibility of a regulatory population of cells present within the spleen cell mixture has not been ruled out. Regulatory T cells are known to have potent immunomodulatory effects on islet graft rejection (18-21), and could possibly account for the delayed graft rejection observed following transfer of tolerant spleen cells.

Islet graft infiltration was characteristic of those mice that rejected their grafts. Heavy infiltration of $CD4^+$ T cells could be detected at the site of rejected grafts, with $CD8^+$ T cells and $CD11b^+$ cells also present, but to a lesser extent, indicating the importance of helper T cells in mediating graft rejection. BALB/c rag-/- mice that were transferred with tolerant spleen cells, in general, displayed a reduced intensity of staining for CD4, CD8 and CD11b. The mouse which received tolerant spleen cells, and

maintained normoglycemia for greater than 50 days displayed a markedly reduced infiltration of the islet graft.

Mouse anti-rat antibody production was closely correlated to graft function as the serum of mice that rejected their xenografts contained higher levels of anti-rat antibodies. Conversely, the sera of the mouse with graft function after 50 days post-adoptive transfer contained virtually no increase in anti-rat antibodies compared to naïve untransplanted controls. Given this data, it would appear likely that both cellular and humoral mediated immune responses participate in the rejection of the islet grafts in this experimental model.

To further examine whether rapamycin and anti-LFA-1 anti-rejection therapy was inducing tolerance, we nephrectomized mice that were treated with rapamycin and anti-LFA-1 and maintained islet graft function for >100 days, and re-transplanted with a WF or Lewis rat islet graft. Interestingly, 100% of mice (4/4) were tolerant of a second WF rat islet graft and maintained normoglycemia for over 100 days following the second transplant. In contrast however, as of the time of writing, only 33% of recipients (1/3) who received Lewis rat islets at the time of the second transplant maintained normoglycemia. In contrast to our adoptive transfer experiment, tolerance in this experimental model appears to be not just species specific, but strain specific as well. There are several factors which could explain this discrepancy. The possibility exists that the spleen in mice treated with rapamycin and anti-LFA-1 may not contain a significant level of regulatory cells, which could be required for the protection of the islet graft. It has been shown that tolerance which is at least partly induced via the presence of regulatory T cells occurs locally, at the site of the graft and in the regional lymph nodes

(18;22;23). Therefore, by transferring cells from the spleen, we may only be transferring peripheral cells which are not tolerant of the islet graft. Another possible explanation of this discrepancy is based on the finding that extensive T-cell proliferation can occur under conditions of lymphopenia. This process which is termed homeostatic proliferation has been found to induce the acquisition of functional memory T cells which play a role in the prevention of tolerance induction (24-26). BALB/c rag-/- mice, which lack T and B cells, are classified as lymphopenic. It could be hypothesized that following adoptive transfer of donor spleen cells, that these cells undergo homeostatic proliferation, creating a barrier to the induction of tolerance.

Pig islets represent a potential source of islets for transplantation in humans. Many experimental models utilize mice as pig islet recipients, which represents a phylogenetically disparate relationship. It has been well documented that as the degree of phylogenetic disparity increases between a donor and recipient, the indirect pathway of antigen presentation dominates (27;28). As pigs are more closely related to humans (5), the participation of the direct pathway of immune activation becomes increasingly active. Therefore anti-rejection therapies will need to be efficacious in both direct and indirect antigen presentation for pig islet xenotransplantation to succeed in the clinic. Rat islet xenotransplantation into mice offers unique model for studying effective anti-rejection therapies in concordant islet xenotransplantation. Rapamycin and anti-LFA-1 short-term therapy appears to be extremely efficacious at inducing indefinite islet xenograft survival as well as inducing tolerance to WF rat islet grafts. Further experiments are required to determine if tolerance is induced through a regulatory T cell population, however several studies have shown that rapamycin therapy can induce and selectively expand

$CD4^+CD25^+Foxp3^+$ regulatory T cells (6;8). The results of this study indicate that rapamycin and anti-LFA-1 therapy could form a significant component of future anti-rejection regimens aimed at inducing tolerance in clinical xenogeneic islet transplantation.

3.5 REFERENCES

1. Ryan,E.A., Paty,B.W., Senior,P.A., Bigam,D., Alfadhli,E., Kneteman,N.M., Lakey,J.R., and Shapiro,A.M. 2005. Five-year follow-up after clinical islet transplantation. *Diabetes* 54:2060-2069.

2. Shapiro,A.M., Lakey,J.R., Ryan,E.A., Korbutt,G.S., Toth,E., Warnock,G.L., Kneteman,N.M., and Rajotte,R.V. 2000. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N.Engl.J.Med.* 343:230-238.

3. Montalbano,M., Neff,G.W., Yamashiki,N., Meyer,D., Bettiol,M., Slapak-Green,G., Ruiz,P., Manten,E., Safdar,K., O'Brien,C. *et al.* 2004. A retrospective review of liver transplant patients treated with sirolimus from a single center: an analysis of sirolimus-related complications. *Transplantation* 78:264-268.

4. Van Gelder,T., ter Meulen,C.G., Hene,R., Weimar,W., and Hoitsma,A. 2003. Oral ulcers in kidney transplant recipients treated with sirolimus and mycophenolate mofetil. *Transplantation* 75:788-791.

5. Tree of Life Web Project, Eutheria. Placental Mammals. The Tree of Life Web Project . 1997.
Ref Type: Electronic Citation

6. Battaglia,M., Stabilini,A., Draghici,E., Gregori,S., Mocchetti,C., Bonifacio,E., and Roncarolo,M.G. 2006. Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance. *Diabetes* 55:40-49.

7. Anderson,K.M. and Zimring,J.C. 2006. Rapamycin prolongs susceptibility of responding T cells to tolerance induction by CD8+ veto cells. *Transplantation* 81:88-94.

8. Battaglia,M., Stabilini,A., and Roncarolo,M.G. 2005. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 105:4743-4748.

9. Battaglia,M., Stabilini,A., Draghici,E., Migliavacca,B., Gregori,S., Bonifacio,E., and Roncarolo,M.G. 2006. Induction of Tolerance in Type 1 Diabetes via Both CD4+CD25+ T Regulatory Cells and T Regulatory Type 1 Cells. *Diabetes* 55:1571-1580.

10. Perez,O.D., Mitchell,D., Jager,G.C., South,S., Murriel,C., McBride,J., Herzenberg,L.A., Kinoshita,S., and Nolan,G.P. 2003. 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.

11. Nicolls,M.R. and Gill,R.G. 2006. LFA-1 (CD11a) as a therapeutic target. *Am.J.Transplant.* 6:27-36.

12. Grochowiecki,T., Gotoh,M., Dono,K., Takeda,Y., Sakon,M., Yagita,H., Okumura,K., Miyasaka,M., and Monden,M. 2000. Induction of unresponsiveness to islet xenograft by MMC treatment of graft and blockage of LFA-1/ICAM-1 pathway. *Transplantation* 69:1567-1571.

13. Ohta,Y., Gotoh,M., Ohzato,H., Fukuzaki,T., Nishihara,M., Dono,K., Umeshita,K., Sakon,M., Yagita,H., Okumura,K. *et al.* 1998. Direct antigen presentation through binding of donor intercellular adhesion molecule-1 to recipient lymphocyte function-associated antigen-1 molecules in xenograft rejection. *Transplantation* 65:1094-1100.

14. Zeng,Y., Gage,A., Montag,A., Rothlein,R., Thistlethwaite,J.R., and Bluestone,J.A. 1994. Inhibition of transplant rejection by pretreatment of xenogeneic pancreatic islet cells with anti-ICAM-1 antibodies. *Transplantation* 58:681-689.

15. Lebwohl,M., Tyring,S.K., Hamilton,T.K., Toth,D., Glazer,S., Tawfik,N.H., Walicke,P., Dummer,W., Wang,X., Garovoy,M.R. *et al.* 2003. A novel targeted T-cell modulator, efalizumab, for plaque psoriasis. *N.Engl.J.Med.* 349:2004-2013.

16. Korbutt,G.S., Elliott,J.F., Ao,Z., Smith,D.K., Warnock,G.L., and Rajotte,R.V. 1996. Large scale isolation, growth, and function of porcine neonatal islet cells. *J.Clin.Invest* 97:2119-2129.

17. Shapiro,A.M., Lakey,J.R., Ryan,E.A., Korbutt,G.S., Toth,E., Warnock,G.L., Kneteman,N.M., and Rajotte,R.V. 2000. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N.Engl.J.Med.* 343:230-238.

18. Green,E.A., Gorelik,L., McGregor,C.M., Tran,E.H., and Flavell,R.A. 2003. CD4+CD25+ T regulatory cells control anti-islet CD8+ T cells through TGF-beta-TGF-beta receptor interactions in type 1 diabetes. *Proc.Natl.Acad.Sci.U.S.A* 100:10878-10883.

19. Weber,S.E., Harbertson,J., Godebu,E., Mros,G.A., Padrick,R.C., Carson,B.D., Ziegler,S.F., and Bradley,L.M. 2006. Adaptive islet-specific regulatory CD4 T cells control autoimmune diabetes and mediate the disappearance of pathogenic Th1 cells in vivo. *J.Immunol.* 176:4730-4739.

20. Chen,D., Zhang,N., Fu,S., Schroppel,B., Guo,Q., Garin,A., Lira,S.A., and Bromberg,J.S. 2006. CD4+ CD25+ regulatory T-cells inhibit the islet innate immune response and promote islet engraftment. *Diabetes* 55:1011-1021.

21. Saborio,D.V., Chowdhury,N.C., Jin,M.X., Chandraker,A., Sayegh,M.H., and Oluwole,S.F. 1999. Regulatory T cells maintain peripheral tolerance to islet allografts induced by intrathymic injection of MHC class I allopeptides. *Cell Transplant.* 8:375-381.

22. Bour-Jordan,H., Salomon,B.L., Thompson,H.L., Szot,G.L., Bernhard,M.R., and Bluestone,J.A. 2004. Costimulation controls diabetes by altering the balance of pathogenic and regulatory T cells. *J.Clin.Invest* 114:979-987.

23. Nanji,S.A., Hancock,W.W., Luo,B., Schur,C.D., Pawlick,R.L., Zhu,L.F., Anderson,C.C., and Shapiro,A.M. 2006. Costimulation blockade of both inducible costimulator and CD40 ligand induces dominant tolerance to islet allografts and prevents spontaneous autoimmune diabetes in the NOD mouse. *Diabetes* 55:27-33.

24. Hickman,S.P. and Turka,L.A. 2005. Homeostatic T cell proliferation as a barrier to T cell tolerance. *Philos.Trans.R.Soc.Lond B Biol.Sci.* 360:1713-1721.

25. Taylor,D.K., Neujahr,D., and Turka,L.A. 2004. Heterologous immunity and homeostatic proliferation as barriers to tolerance. *Curr.Opin.Immunol.* 16:558-564.

26. Wu,Z., Bensinger,S.J., Zhang,J., Chen,C., Yuan,X., Huang,X., Markmann,J.F., Kassaee,A., Rosengard,B.R., Hancock,W.W. *et al.* 2004. Homeostatic proliferation is a barrier to transplantation tolerance. *Nat.Med.* 10:87-92.

27. Andres,A., Toso,C., Morel,P., Bosco,D., Bucher,P., Oberholzer,J., Mathe,Z., Mai,G., Wekerle,T., Berney,T. *et al.* 2005. Macrophage depletion prolongs discordant but not concordant islet xenograft survival. *Transplantation* 79:543-549.

28. Rayat,G.R., Johnson,Z.A., Beilke,J.N., Korbutt,G.S., Rajotte,R.V., and Gill,R.G. 2003. The degree of phylogenetic disparity of islet grafts dictates the reliance on indirect CD4 T-cell antigen recognition for rejection. *Diabetes* 52:1433-1440.

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

4.1 GENERAL DISCUSSION

Type 1 diabetes mellitus (T1DM) is a chronic metabolic disorder characterized by hyperglycemia, resulting from the autoimmune destruction of the insulin producing β cells of the pancreas. The discovery of insulin in 1921 dramatically changed the treatment of T1DM and transformed what was an acutely fatal illness, into a chronic disease (1-3). While the discovery of insulin was a tremendous leap forward in the treatment of T1DM, it became apparent that exogenous insulin injections could not provide the tight degree of glycemic control found in people with functional β cells. As the life expectancy of patients with T1DM increased, the number of side effects, associated with fluctuating glucose levels, also increased. These side effects can include foot ulceration, retinopathy, neuropathy, and nephropathy, greatly shorten life expectancy, as well as diminish the quality of life, highlighting the need for better treatment alternatives for people with T1DM (4-14).

Several alternative treatment options have emerged for patients with T1DM including: islet transplantation (15;16), pancreas transplantation (17;18), stem cell differentiation (19-22), and β cell regeneration (23;24). Islet transplantation has recently gained worldwide attention as a viable treatment alternative for patients with T1DM. The Edmonton Protocol was the first report of insulin independence in 100% of patients at 1 year following an allogeneic islet transplantation (25). The recent success of human islet transplantation has thrust islet transplantation to the forefront of treatment possibilities for

patients with T1DM. Despite this dramatic advancement, islet transplantation at present is not a viable treatment option for the majority of people with T1DM. There are two primary reasons for this finding, the first being the shortage of donor pancreatic tissue, and the second, the need for continuous immunosuppression to prevent the immune mediated rejection of the transplanted islet graft. These shortcomings have prompted investigators to seek out new sources of insulin producing tissue and new methods of preventing the rejection of the transplanted islets which are not associated with the toxicity of the currently implemented immunosuppressive regimen (26;27). To address the issue of organ shortage, xenogeneic donors have been proposed as a potential source of pancreatic tissue for human islet transplantation. While many xenogeneic sources of insulin producing tissue have been used experimentally, pigs are arguably the most ideal source for humans because of the structural similarity of porcine insulin to human insulin (28), the potentially unlimited supply of porcine tissue (29), and the potential to decrease the immunogenicity of the tissue by genetic manipulation (30;31). However, xenogeneic islets elicit a strong immune response. This has prompted investigation of new ways of preventing xenogeneic islet rejection that can be given short term and induce tolerance to the xenogeneic islets, abrogating the toxicity associated with chronic immunosuppression.

Rapamycin (sirolimus) an immunosuppressive drug, is one of the mainstays of treatment in the Edmonton Protocol (32). Rapamycin has a potent ability to inhibit T and B cell proliferation (33-36), which has made it the therapy of choice in many different types of transplantation (37). Immunosuppressive drugs historically, have been thought to act as a barrier to tolerance induction. Several studies however, have suggested that

rapamycin may actually promote the induction of antigen specific tolerance (38;39) as well as selectively expand $CD4^+CD25^+Foxp3^+$ regulatory T cells (40), prevent new onset diabetes and induce immune tolerance in NOD mice (41).

Leukocyte function associated antigen-1 (LFA-1) has emerged as an important contributor of T cell activation. LFA-1 facilitates the migration of leukocytes to the site of an immune response (42), stabilizes the APC/T cell immune synapse (43), and produces its own positive activation signal (44). The targeting of recipient LFA-1 and donor ICAM-1 has been shown to prolong concordant islet xenotransplantation (45;46), and in discordant islet transplantation the ICAM-1/LFA-1 interaction was found to be critical for xenograft rejection (47). Recently, a humanized form of anti-LFA-1, Efalizumab was approved for phase IV trials for the treatment of psoriasis (48).

While rats do not represent a potential source of islets for clinical transplantation, they do represent an attractive source of islets to study concordant xenogeneic islet transplantation in mice. Porcine islets have been proposed as a suitable alternative source of islets in clinical transplantation (29;49-51). However, at present, many experimental models involving porcine tissue utilize mice as recipients, which represents a discordant relationship (52). Pigs and humans are more closely related than pigs and mice (53), consequently, the type of immune response which is elicited in each situation is different (54;55). If porcine islets are to become a source of islets in clinical transplantation, the immune response must be examined as the relation between donor and recipient becomes more concordant. Analyzing the potential of clinical therapies to prevent xenograft rejection and their ability to prevent concordant and discordant rejection is of significant importance.

Xenogeneic islets are subject to a robust immune response. However in Chapter 2 of this manuscript, it was demonstrated that a short-term administration of anti-LFA-1 alone or in conjunction with rapamycin, prevented the rejection of Wistar-Furth rat islet xenografts in BALB/c mice long-term or >100 days. Islet grafts from mice surviving long-term revealed robust insulin positive cells and a reduction in the extent of immune cell infiltration including reduced levels of $CD4^+$ T cells, $CD8^+$ T cells and $CD11b^+$ cells. The effect of rapamycin and anti-LFA-1 on the humoral immune system was also examined. While treatment with rapamycin did slightly decrease mouse anti-rat antibody levels, anti-LFA-1 therapy alone or in conjunction with rapamycin, significantly reduced mouse anti-rat antibody production to levels similar to untransplanted naïve controls. In addition, the potent efficacy of rapamycin, anti-LFA-1 or both, to completely abrogate the proliferation of BALB/c lymphocytes was demonstrated *in vitro*. Chapter 2 revealed the effectiveness of targeting LFA-1 in concordant xenogeneic islet transplantation as this monotherapy was as effective as combining it with rapamycin. Given the data shown, it appears likely that the prevention of islet graft rejection following these anti-rejection therapies is attributed to the ability to target both the cell mediated and humoral immune responses, with an emphasis on the humoral immune response. What was unclear at the conclusion of Chapter 2 was whether the combination therapy was merely prolonging graft function or whether it was inducing tolerance to the rat islet xenografts.

In Chapter 3 the ability of rapamycin and anti-LFA-1 short-term therapy to induce tolerance to rat islet xenografts was examined. Despite the observation that anti-LFA-1 monotherapy proved to be equally as efficacious as combining with rapamycin, the combination therapy of rapamycin and anti-LFA-1 was studied in Chapter 3, given recent

findings that rapamycin treatment selectively expands $CD4^+CD25^+Foxp3^+$ regulatory T cells (40) and induces allogeneic islet graft tolerance (38). The combination therapy was administered short-term and was found to induce indefinite islet xenograft survival with 100% of mice maintaining xenograft function for over 200 days post-transplantation. The spleen cells of combination treated recipients once adoptively transferred to BALB/c rag-/- mice, did demonstrate a delay in the rejection of the islet graft compared to naïve spleen cells, although the difference was not significant. Tolerant spleen cells from combination treated BALB/c mice were equally as effective at rejecting Lewis rat islet grafts in BALB/c rag-/- mice. When the tolerant spleen cells were combined with naïve spleen cells, the tolerant spleen cells appeared to suppress the ability of the naïve spleen cells to acutely reject the islet graft, although this difference was not significant. Despite the observation that the majority of mice rejected their islet xenografts once adoptively transferred, one mouse from each of the tolerant spleen cell, and naïve combined with tolerant spleen cell groups maintained islet graft function for greater than 50 days after transfer. This finding suggests that the tolerance induced via rapamycin and anti-LFA-1 therapy is incomplete. While it could not be determined definitively, the tolerant spleen cells appeared to be species specific as the tolerant spleen cells rejected C57BL/6 islet grafts as effectively as naïve spleen cells rejected WF-rat grafts. A lack of insulin staining and islet graft infiltration with $CD4^+$ T cells, and to a lesser extent $CD8^+$T cells, $CD11b^+$ cells was characteristic of rejected islet grafts, as was an increase in the production of mouse anti-rat antibodies. However, insulin positive staining was still detected in one mouse receiving tolerant spleen cells with graft function at 50 days. In addition, reduced staining for $CD4^+$ T cells, $CD8^+$ T cells, and $CD11b^+$ was detected, as

well as a reduction in mouse anti-rat antibody, compared to mice adoptively with naïve spleen cells. The ability of rapamycin and anti-LFA-1 to induce tolerance was next examined in an animal model with an intact adaptive immune system. BALB/c mice that were transplanted with WF rat islets and received rapamycin and anti-LFA-1 were tolerant of a second WF rat islet graft, maintaining graft function for greater than 100 days following a second islet transplantation. Interestingly, the majority of BALB/c mice were not tolerant of a second Lewis rat islet transplant, indicating that rapamycin and anti-LFA-1 therapy induces strain specific tolerance. This discrepancy from the adoptive transfer experiment could be explained by the possibility that the spleen in mice treated with rapamycin and anti-LFA-1 may not contain a significant level of regulatory cells, which could be required for the protection of the islet graft. Therefore, by transferring cells from the spleen we may only be transferring peripheral cells, which are not tolerant of the islet graft. Another possible explanation of this discrepancy could be that following adoptive transfer of donor spleen cells, these cells may undergo homeostatic proliferation, creating a barrier to the induction of tolerance. Clearly more experimentation is needed to demonstrate the reproducibility of these observations, as well as determine whether anti-LFA-1 monotherapy is equally as effective at inducing tolerance in this experimental model.

4.2 CONCLUSION

The success of the Edmonton Protocol has provided tremendous support for islet transplantation as a potential alternative therapy for patients with T1DM. However, despite these recent advancements in islet transplantation, this therapy is not available to

the vast majority of patients with T1DM because of the shortage of donor islets and the toxicity of the immunosuppressive regimen. Xenogeneic islet transplantation holds promise for clinical transplantation because of the potentially unlimited supply of islets. As xenotransplantation approaches clinic trials, renewed concerns over effective anti-rejection therapies in xenogeneic islet transplantation are emerging. For xenotransplantation to succeed clinically, demonstrated safety, and more effective anti-rejection protocols will need to be developed. We have shown that a short-term anti-rejection therapy of rapamycin and anti-LFA-1 can successfully prolong concordant xenogeneic islet grafts indefinitely and induce tolerance to these same islet grafts. It is the hope that the research presented in this manuscript will provide insight into the nature of the immune response to xenogeneic islet transplantation in humans and aid in the development of effective, tolerance inducing therapies, so that patients with T1DM will once again know a life free from their disease.

4.3 REFERENCES

1. Pickup JC and Williams G. 2003. Textbook of Diabetes. Blackwell, Malden.
2. King,K.M. 2003. A history of insulin: from discovery to modern alternatives. *Br.J.Nurs.* 12:1137-1141.
3. King,K.M. and Rubin,G. 2003. A history of diabetes: from antiquity to discovering insulin. *Br.J.Nurs.* 12:1091-1095.
4. Turina,M., Christ-Crain,M., and Polk,H.C., Jr. 2005. Impact of diabetes mellitus and metabolic disorders. *Surg.Clin.North Am.* 85:1153-61, ix.
5. Tripathi,B.K. and Srivastava,A.K. 2006. Diabetes mellitus: Complications and therapeutics. *Med.Sci.Monit.* 12:RA130-RA147.
6. Russell,T.A. 2006. Diabetic nephropathy in patients with type 1 diabetes mellitus. *Nephrol.Nurs.J.* 33:15-28.

7. Bober,E. and Buyukgebiz,A. 2005. Hypoglycemia and its effects on the brain in children with type 1 diabetes mellitus. *Pediatr.Endocrinol.Rev.* 2:378-382.

8. Perkins,B.A. and Krolewski,A.S. 2005. Early nephropathy in type 1 diabetes: a new perspective on who will and who will not progress. *Curr.Diab.Rep.* 5:455-463.

9. Haller,M.J., Atkinson,M.A., and Schatz,D. 2005. Type 1 diabetes mellitus: etiology, presentation, and management. *Pediatr.Clin.North Am.* 52:1553-1578.

10. Marre,M. 2002. Nephropathy in type 1 diabetes. *Semin.Vasc.Med.* 2:97-107.

11. Shore,A.C. 2002. The microvasculature in type 1 diabetes. *Semin.Vasc.Med.* 2:9-20.

12. Rossing,P. 2005. The changing epidemiology of diabetic microangiopathy in type 1 diabetes. *Diabetologia* 48:1439-1444.

13. Libby,P., Nathan,D.M., Abraham,K., Brunzell,J.D., Fradkin,J.E., Haffner,S.M., Hsueh,W., Rewers,M., Roberts,B.T., Savage,P.J. *et al.* 2005. Report of the National Heart, Lung, and Blood Institute-National Institute of Diabetes and Digestive and Kidney Diseases Working Group on Cardiovascular Complications of Type 1 Diabetes Mellitus. *Circulation* 111:3489-3493.

14. Dahl-Jorgensen,K., Larsen,J.R., and Hanssen,K.F. 2005. Atherosclerosis in childhood and adolescent type 1 diabetes: early disease, early treatment? *Diabetologia* 48:1445-1453.

15. Ryan,E.A., Paty,B.W., Senior,P.A., Bigam,D., Alfadhli,E., Kneteman,N.M., Lakey,J.R., and Shapiro,A.M. 2005. Five-year follow-up after clinical islet transplantation. *Diabetes* 54:2060-2069.

16. Shapiro,A.M., Lakey,J.R., Paty,B.W., Senior,P.A., Bigam,D.L., and Ryan,E.A. 2005. Strategic opportunities in clinical islet transplantation. *Transplantation* 79:1304-1307.

17. Cohen,D.J., St Martin,L., Christensen,L.L., Bloom,R.D., and Sung,R.S. 2006. Kidney and pancreas transplantation in the United States, 1995-2004. *Am.J.Transplant.* 6:1153-1169.

18. Humar,A., Kandaswamy,R., Granger,D., Gruessner,R.W., Gruessner,A.C., and Sutherland,D.E. 2000. Decreased surgical risks of pancreas transplantation in the modern era. *Ann.Surg.* 231:269-275.

19. Meier,J.J., Bhushan,A., and Butler,P.C. 2006. The potential for stem cell therapy in diabetes. *Pediatr.Res.* 59:65R-73R.

20. Madsen,O.D. 2005. Stem cells and diabetes treatment. *APMIS* 113:858-875.

21. Roche,E., Enseat-Wase,R., Reig,J.A., Jones,J., Leon-Quinto,T., and Soria,B. 2006. Therapeutic potential of stem cells in diabetes. *Handb.Exp.Pharmacol.*147-167.

22. Otonkoski,T., Gao,R., and Lundin,K. 2005. Stem cells in the treatment of diabetes. *Ann.Med.* 37:513-520.

23. Yamada,S. and Kojima,I. 2005. Regenerative medicine of the pancreatic beta cells. *J.Hepatobiliary.Pancreat.Surg.* 12:218-226.

24. Trucco,M. 2005. Regeneration of the pancreatic beta cell. *J.Clin.Invest* 115:5-12.

25. Shapiro,A.M., Lakey,J.R., Ryan,E.A., Korbutt,G.S., Toth,E., Warnock,G.L., Kneteman,N.M., and Rajotte,R.V. 2000. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N.Engl.J.Med.* 343:230-238.

26. Jonathan,R.T., Mohammadreza,M., and Shapiro,A.M. 2006. Current status of clinical islet cell transplantation. *Methods Mol.Biol.* 333:47-104.

27. Merani,S. and Shapiro,A.M. 2006. Current status of pancreatic islet transplantation. *Clin.Sci.(Lond)* 110:611-625.

28. O'Connell,P. 2002. Pancreatic islet xenotransplantation. *Xenotransplantation.* 9:367-371.

29. Korbutt,G.S., Elliott,J.F., Ao,Z., Smith,D.K., Warnock,G.L., and Rajotte,R.V. 1996. Large scale isolation, growth, and function of porcine neonatal islet cells. *J.Clin.Invest* 97:2119-2129.

30. Phelps,C.J., Koike,C., Vaught,T.D., Boone,J., Wells,K.D., Chen,S.H., Ball,S., Specht,S.M., Polejaeva,I.A., Monahan,J.A. *et al.* 2003. Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science* 299:411-414.

31. Dor,F.J., Tseng,Y.L., Cheng,J., Moran,K., Sanderson,T.M., Lancos,C.J., Shimizu,A., Yamada,K., Awwad,M., Sachs,D.H. *et al.* 2004. alpha1,3-Galactosyltransferase gene-knockout miniature swine produce natural cytotoxic anti-Gal antibodies. *Transplantation* 78:15-20.

32. Shapiro,A.M., Lakey,J.R., Ryan,E.A., Korbutt,G.S., Toth,E., Warnock,G.L., Kneteman,N.M., and Rajotte,R.V. 2000. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N.Engl.J.Med.* 343:230-238.

33. Asnaghi,L., Bruno,P., Priulla,M., and Nicolin,A. 2004. mTOR: a protein kinase switching between life and death. *Pharmacol.Res.* 50:545-549.

34. Inoki,K., Ouyang,H., Li,Y., and Guan,K.L. 2005. Signaling by target of rapamycin proteins in cell growth control. *Microbiol.Mol.Biol.Rev.* 69:79-100.

35. Kwon,G., Marshall,C.A., Pappan,K.L., Remedi,M.S., and McDaniel,M.L. 2004. Signaling elements involved in the metabolic regulation of mTOR by nutrients, incretins, and growth factors in islets. *Diabetes* 53 Suppl 3:S225-S232.

36. Rowinsky,E.K. 2004. Targeting the molecular target of rapamycin (mTOR). *Curr.Opin.Oncol.* 16:564-575.

37. Meier-Kriesche,H.U., Li,S., Gruessner,R.W., Fung,J.J., Bustami,R.T., Barr,M.L., and Leichtman,A.B. 2006. Immunosuppression: evolution in practice and trends, 1994-2004. *Am.J.Transplant.* 6:1111-1131.

38. Battaglia,M., Stabilini,A., Draghici,E., Gregori,S., Mocchetti,C., Bonifacio,E., and Roncarolo,M.G. 2006. Rapamycin and interleukin-10 treatment induces T regulatory type 1 cells that mediate antigen-specific transplantation tolerance. *Diabetes* 55:40-49.

39. Anderson,K.M. and Zimring,J.C. 2006. Rapamycin prolongs susceptibility of responding T cells to tolerance induction by CD8+ veto cells. *Transplantation* 81:88-94.

40. Battaglia,M., Stabilini,A., and Roncarolo,M.G. 2005. Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 105:4743-4748.

41. Battaglia,M., Stabilini,A., Draghici,E., Migliavacca,B., Gregori,S., Bonifacio,E., and Roncarolo,M.G. 2006. Induction of Tolerance in Type 1 Diabetes via Both CD4+CD25+ T Regulatory Cells and T Regulatory Type 1 Cells. *Diabetes* 55:1571-1580.

42. Shamri,R., Grabovsky,V., Gauguet,J.M., Feigelson,S., Manevich,E., Kolanus,W., Robinson,M.K., Staunton,D.E., von Andrian,U.H., and Alon,R. 2005. Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nat.Immunol.* 6:497-506.

43. Shimizu,Y. 2003. LFA-1: more than just T cell Velcro. *Nat.Immunol.* 4:1052-1054.

44. Perez,O.D., Mitchell,D., Jager,G.C., South,S., Murriel,C., McBride,J., Herzenberg,L.A., Kinoshita,S., and Nolan,G.P. 2003. 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.

45. Grochowiecki,T., Gotoh,M., Dono,K., Takeda,Y., Sakon,M., Yagita,H., Okumura,K., Miyasaka,M., and Monden,M. 2000. Induction of unresponsiveness

to islet xenograft by MMC treatment of graft and blockage of LFA-1/ICAM-1 pathway. *Transplantation* 69:1567-1571.

46. Ohta,Y., Gotoh,M., Ohzato,H., Fukuzaki,T., Nishihara,M., Dono,K., Umeshita,K., Sakon,M., Yagita,H., Okumura,K. *et al.* 1998. Direct antigen presentation through binding of donor intercellular adhesion molecule-1 to recipient lymphocyte function-associated antigen-1 molecules in xenograft rejection. *Transplantation* 65:1094-1100.

47. Zeng,Y., Gage,A., Montag,A., Rothlein,R., Thistlethwaite,J.R., and Bluestone,J.A. 1994. Inhibition of transplant rejection by pretreatment of xenogeneic pancreatic islet cells with anti-ICAM-1 antibodies. *Transplantation* 58:681-689.

48. Lebwohl,M., Tyring,S.K., Hamilton,T.K., Toth,D., Glazer,S., Tawfik,N.H., Walicke,P., Dummer,W., Wang,X., Garovoy,M.R. *et al.* 2003. A novel targeted T-cell modulator, efalizumab, for plaque psoriasis. *N.Engl.J.Med.* 349:2004-2013.

49. Koulmanda,M., Qipo,A., Smith,R.N., and Auchincloss,H., Jr. 2003. Pig islet xenografts are resistant to autoimmune destruction by non-obese diabetic recipients after anti-CD4 treatment. *Xenotransplantation.* 10:178-184.

50. MacKenzie,D.A., Hullett,D.A., and Sollinger,H.W. 2003. Xenogeneic transplantation of porcine islets: an overview. *Transplantation* 76:887-891.

51. Tang,T.H., Li,C.L., Li,X., Jiang,F.Q., Zhang,Y.K., Ren,H.Q., Su,S.S., and Jiang,G.S. 2004. Immune tolerance in pancreatic islet xenotransplantation. *World J.Gastroenterol.* 10:1457-1461.

52. Takahashi,K., Rooney,A.P., and Nei,M. 2000. Origins and divergence times of mammalian class II MHC gene clusters. *J.Hered.* 91:198-204.

53. Tree of Life Web Project, Eutheria. Placental Mammals. The Tree of Life Web Project . 1997.
Ref Type: Electronic Citation

54. Koulmanda,M., Laufer,T.M., Auchincloss,H., Jr., and Smith,R.N. 2004. Prolonged survival of fetal pig islet xenografts in mice lacking the capacity for an indirect response. *Xenotransplantation.* 11:525-530.

55. Rayat,G.R., Johnson,Z.A., Beilke,J.N., Korbutt,G.S., Rajotte,R.V., and Gill,R.G. 2003. The degree of phylogenetic disparity of islet grafts dictates the reliance on indirect CD4 T-cell antigen recognition for rejection. *Diabetes* 52:1433-1440.