University of Alberta

Tolerance to Neonatal Porcine Islet Xenografts Induced by a Combination of Monoclonal Antibodies

by

Hossein Arefanian

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

Doctor of Philosophy
in
Experimental Surgery

Department of Surgery

Fall 2009
Edmonton, Alberta

Examining Committee

Dr. G.R. Rayat, Surgery

Dr. G.S. Korbutt, Surgery

Dr. R.V. Rajotte, Surgery

Dr. C.J. Field, Agricultural, Food & Nutritional Science

Dr. B.J. Hering, Surgery, University of Minnesota

This thesis is dedicated to my wonderful wife, Nafiseh, and my daughters, Mohadeseh, Sama and Sana whose patience with my labours has been divine, and whose love and support has given me the strength of mind and will to accomplish all things.

ABSTRACT

Islet transplantation is a more physiological way to treat type 1 diabetes. However, shortage of donor tissue and chronic administration of immune suppressive drugs has limited the widespread application of this therapy for all patients with type 1 diabetes, particularly children suffering from this disease. Xenogeneic islet transplantation particularly neonatal porcine islets (NPI) holds promise for clinical transplantation because of the potentially unlimited supply of islets. New evidence suggests that monoclonal antibodies (mAbs) specific for immune cell surface molecules could be employed in the prevention of islet graft rejection as well as induction of immunological tolerance to the transplanted grafts without the need for continuous administration of harmful immune suppressive drugs. It was shown by our group that short-term administrations of a combination of anti-LFA-1 and anti-CD154 mAbs which targets both adhesion and costimulatory pathways of T cell activation, is highly effective in preventing NPI xenograft rejection. In this thesis, we determined whether short-term administration of a combination of anti-LFA-1 and anti-CD154 mAbs could induce tolerance to NPI xenografts. Our data show that this combination of mAbs can induce dominant, species and tissue specific tolerance to NPI xenografts which is mediated by regulatory T cells in non-autoimmune prone B6 mice. We also found that T cell subsets such as $CD4^+$ and $CD8^+$ T cells as well as antigen presenting cells (APC) play an important role in the induction and maintenance of tolerance to NPI xenografts. In addition we found that PD-1/PDL interaction is

important for induction and maintenance of tolerance to NPI xenografts. Finally, we found that this combined mAb therapy was effective in preventing NPI xenografts rejection in autoimmune prone NOD mice when it was combined with anti-CD4 mAb. It is may hope that the research presented in this thesis will provide insight into the nature of the immune responses 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.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor, Dr. Gina R. Rayat for giving me the chance 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. 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 the 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. I would like to thank Dr. Catherine Field for her insight, comments and critical review of this manuscript. Finally I would like to thank Dr. Bernhard Hering for making the time to critically review this manuscript and be a part of my final defence committee. Several faculty members within the department of Surgery and Alberta Diabetes Institute have provided me with valuable guidance and I would like to specifically thank Dr. Ron Gill, Dr. Colin Anderson, Dr. Ted Tredget, and Dr. Tom Churchill for their advice and support.

I would like to extend a special thanks to members of our lab. Thanks to Eric Tredget who has become a wonderful friend of mine. Thank you to Dr. Dereck Mok, Leilani Langer, and Qahir Ramji for their friendship and advice. I

would like to acknowledge and thank our wonderful summer students: Raissa Petriw, Stephanie Wilkes, Matt Sadler, Kailey Winton, Amogh Agrawal, Victoria Tian, Connie Le, Kathryn Chevalier, Brendan Sigalet, Serge Goekjian, and Scott Mackay for all their hard work. I wish them all the best in the future.

Thank you to Rosemarie Henley, Margaret Henley and Colleen Ruptash the staff of Surgical Medical Research Institute and Alberta Diabetes Institute who have always gone above and beyond for me. I owe Deb Dixon special thanks who have been an invaluable part of my project and a wonderful teacher. She is another wonderful and trusted friend of mine. I wish her a lifetime of happiness, whatever road she chooses. I would like to thank Dawne Colwell for her wonderful artistry, which has been instrumental in my posters, presentations and this manuscript.

I am also very grateful to the Iranian Ministry of Health and Medical Education and Muttart Diabetes Research & Training Center, the Department of Surgery and the Faculty of Graduate Studies and Research for their financial support.

Finally, I would like to thank my parents who have supported me in every way, and for ingraining within me the desire to learn and achieve, even from an early age. To my children, I express a deep love and gratitude for patience as Daddy has spent many hours locked away in the lab, and for making our home the most wonderful place to be. Most of all, I would like to thank Nafiseh, my wonderful wife, who has made many sacrifices to allow me to pursue my research and academic endeavours. I will forever be appreciative of the support and

inspiration you have given me. Finally, I thank Allah for the opportunities, talents and blessings that I have been granted, and for the strength provided unto me in times of need.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

αGal	Galactosyl alpha (1-3) galactose
Ab	Antibody
ABC/HP	Avidin-biotin complex/horseradish peroxidase
ADCC	Antibody-dependent cell mediated cytotoxicity
ANOVA	Analysis of variance
APC	Antigen presentation cell
ATP	Adenosine triphosphate
B6	C57BL/6J
BB rat	Bio-breeding rat
BG	Blood glucose
BSA	Bovine serum albumin
BTLA	B and T lymphocyte attenuator
BW	Body weight
CCR	CC chemokine receptors
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CK	Cytokeratin
ConA	Concanavalin A
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DAB	3,3-diaminobenzidinetetrahydrochloride
DC	Dendritic cell

DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DSG	15-deoyspergualin
EGF	Epidermal growth factor
EGTA	Ethylene glycol-bis (2-aminoethyl)-n,n,n’,n’-tetraacetic acid
ELISA	Enzyme linked immunosorbant assay
ESC	Embryonic stem cell
FACS	Florescence activated cell sorter
FBS	Fetal bovine serum
FICC	Fetal islet-like cell cluster
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box p3
GAPDH	Glyceraldehydes 3-phosphate dehydrogenase
GITR	Glucocorticoid-induced tumor necrosis factor receptor
GLP	Good laboratory practice
GLUT-4	Glucose transporter-4
GMP	Good manufacturing practice
GnT-III	N-acetylglucosaminyltransferase-iii
HAR	Hyper acute rejection
HBSS	Hank’s balanced salt solution
HLA	Human leukocyte antigen
i.p.	Intraperitoneal
IAA	Insulin auoantibody
IBMIR	Instant blood mediated inflammatory reaction
ICAM	Intercellular adhesion molecule

ICOS	Inducible T cell costimulator
IE	Islet equivalent
IFG-γ	Interferon-gamma
Ig	Immunoglobulin
IgG	Immunoglobulin g
IL	Interleukin
JAK	Janus kinase
JAM-1	Junctional adhesion molecule-1
kD	Kilodalton
LFA-1	Leukocyte function associated antigen-1
mAb	Monoclonal antibody
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor κB
NK	Natural killer
NKT	Natural killer T-cells
NOD	Non-obese diabetic
NPI	Neonatal porcine islets
OCT	Optimal cutting temperature
OGTT	Oral glucose tolerance test
P/S	Penicillin/streptomycin
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PD-1	Programmed death-1
PD-L	Programmed death-1 ligand
Pdx-1	Pancreatic and duodenal homeobox-1
PERV	Porcine endogenous retrovirus
PP cell	Polypeptide cell
rag	Recombination activating gene
RNA	Ribonucleic acid
RT	Reverse transcriptase
SCID	Severe combined immunodeficiency
SEM	Standard error of mean
SLA	Swine leukocyte antigen
TCR	T cell receptor
TGF β	Transforming growth factor beta
T_H	T helper
TNF-α	Tumor necrosis factor-alpha
TRAF	Tumor necrosis factor receptor-associated factor
WHO	World health organization
XNA	Xenoreactive natural antibody

CHAPTER 1

GENERAL INTRODUCTION

1.1. DIABETES MELLITUS

Diabetes mellitus (DM) is a chronic metabolic disease, which is characterized by high blood glucose levels termed as “hyperglycemia”. DM develops due to a lack or low production of insulin, or resistance to the effect of insulin (1,2).

1.1.1. History Of DM

The term “diabetes” is derived from the Greek word meaning “siphon” and was first coined by Aretaeus of Cappadocia in the 2nd century AD (1). This name was formed from the prefix *dia-*, “across, apart” and the verb *bainein*, “to walk, stand”. The word "siphon" gave rise to the use of diabetes as the name for a disease involving the discharge of excessive amounts of urine (1). 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). Hindu physician Sushrut (6th century BC) identified diabetes with obesity and sedentary lifestyle, advising exercises to help cure it (1,2). He was believed to be the first to recognize the sweetness of diabetic urine by observing that ants were attracted to a diabetic person's urine, and called the disease “sweet urine disease”. A Persian physician, Avicenna (980-1037) provided a detailed account on diabetes mellitus in “The Canon of Medicine” describing the abnormal appetite and the collapse of sexual functions and he documented the sweet taste of diabetic urine (3). Diabetes is first recorded in English, in the form “diabetes”, in a medical text written around 1425. In 1675,

Thomas Willis added the word mellitus, from the Latin meaning “honey”, a reference to the sweet taste of the urine (1,2). In 1776, Matthew Dobson confirmed that the sweet taste was because of an excess of a kind of sugar in the urine and blood of people with diabetes. Although diabetes has been recognized since antiquity, pathogenesis of diabetes has only been understood experimentally since about 1900. The role for the pancreas in diabetes was described by Joseph von Mering and Oskar Minkowski, who in 1889 found that dogs whose pancreas was removed developed all the signs and symptoms of diabetes and died shortly afterwards (1,2). In 1910, Sir Edward Albert Sharpey-Schafer suggested that people with diabetes were deficient in a single chemical that was normally produced by the pancreas and he proposed calling this substance “insulin”, from the *insula*, meaning island, in reference to the insulin-producing islets of Langerhans in the pancreas coined by Laguesse in 1899 (4). The endocrine role of the pancreas in metabolism, and indeed the existence of insulin, was not further clarified until 1921, when Sir Frederick Grant Banting and Charles Herbert Best repeated the work of Von Mering and Minkowski, and went further to demonstrate they could reverse induced diabetes in dogs by giving them an extract from the pancreatic islets of Langerhans of healthy dogs. Banting, Best, and colleagues (especially the chemist James Collip and J.J.R Macleod) went on to purify the hormone insulin from bovine pancreases at the University of Toronto (1,5,6). This led to the availability of an effective treatment, insulin injections, and the first patient was treated in 1922. For this, Banting and laboratory director MacLeod received the Nobel Prize in Physiology or Medicine in 1923; both

shared their Prize money with others in the team who were not recognized, in particular Best and Collip. Banting and Best made the patent available without charge and did not attempt to control commercial production. Insulin production and therapy rapidly spread around the world, largely as a result of this decision. Banting is honored by World Diabetes Day which is held on his birthday, November 14th. The distinction between what is now known as type 1 diabetes and type 2 diabetes was first clearly made by Sir Harold Percival (Harry) Himsworth, and published in January 1936 (1,5,6).

1.1.2. Epidemiology of DM

The incidence of DM is increasing rapidly. Currently close to 246 million people suffer from diabetes worldwide, it is expected to raise this number to 380 million by 2025 (7). Diabetes mellitus occurs throughout the world, but is more common (especially type 2) in the more developed countries. The greatest increase in prevalence is, however, expected to occur in Asia and Africa, where most patients will probably be found by 2030 (8). For at least 20 years, diabetes rates in North America have been increasing substantially. In Canada the number of patient with diabetes is expected to increase from the 2 million current patients to approximately 3 million patients by 2010 (7). In 2008 there were about 24 million people with diabetes in the United States alone, from those 5.7 million people remain undiagnosed (8). The increase in incidence of diabetes in developing countries follows the trend of urbanization and lifestyle changes, perhaps most importantly a "Western-style" diet. This has suggested an

environmental (i.e., dietary) effect, but there is little understanding of the mechanism(s) at present, though there is much speculation, some of it most compellingly presented. The American Diabetes Association cite the 2003 assessment that 1 in 3 Americans born after 2000 will develop diabetes in their lifetime (8). Diabetes mellitus prevalence increases with age, and the numbers of older persons with diabetes are expected to grow as the elderly population increases in number. The National Health and Nutrition Examination Survey (NHANES III) demonstrated that, in the population over 65 years old, 18% to 20% have diabetes, with 40% having either diabetes or its precursor form of "impaired glucose tolerance" (9). According to the American Diabetes Association, approximately 23.1% (12.2 million) of Americans age 60 and older have diabetes (8). Indigenous populations in first world countries have a higher prevalence and increasing incidence of diabetes than their corresponding non-indigenous populations. In Australia the age-standardized prevalence of self-reported diabetes in Indigenous Australians is almost 4 times that of non-indigenous Australians. About 5%–10% of diabetes cases in North America are type 1, with the rest being type 2. The fraction of type 1 in other parts of the world differs; this is probably due to both differences in the rate of type 1 and differences in the rate of other types, most prominently type 2. Most of this difference is not currently understood (8).

1.1.3. The Impact of DM

DM has a negative impact on both individual patients and society as a whole. It appears to have been a death sentence in the ancient era. Hippocrates makes no mention of it, which may indicate that he felt the disease was incurable. Aretaeus did attempt to treat it but could not give a good prognosis; he commented that "life (with diabetes) is short, disgusting and painful" (2,5,6). At a personal level, patients with diabetes mostly have a low level of quality of life because they need to modify their diets, monitor the blood glucose level, and administer of insulin medications continuously, in addition to the risk of complications and chronic diseases which has direct effect on the economical and social aspects of their daily lives (10,11). Chronic hyperglycemia and fluctuation of blood glucose level can result in two types of secondary complications; acute and chronic complications which can result in the dysfunctions of a number of organ systems such as the kidneys, heart and blood vessels, eyes, and nerves (1,2). Diabetes ketoacidosis (KDA), hyperosmolar non-ketinic syndrome (HNS), hypoglycaemia and diabetic coma considered as acute and dangerous complications that are always a medical emergency. Long-term complications which are known as chronic complications can result in vascular diseases which represent the majority of the morbidity and mortality associated with DM (1,2,10,11). In diabetes, the resulting problems are grouped under "microvascular disease" (due to damage to small blood vessels) and "macrovascular disease" (due to damage to the arteries). The damage to small blood vessels leads to a microangiopathy, which can cause: *i)* Diabetic retinopathy; which can lead to

severe vision loss or blindness, *ii)* Diabetic neuropathy, which can lead to abnormal and decreased sensation, when combined with damaged blood vessels this can lead to diabetic foot which increases rates of skin ulcers and infection and delay wound healing, and in serious cases, necrosis and gangrene. It is the most common cause of non-traumatic adult amputation, usually of toes and or feet, *iii)* Diabetic amyotrophy, which is muscle weakness due to neuropathy, *iv)* Diabetic nephropathy, damage to the kidney which can lead to chronic renal failure, and *v)* Diabetic cardiomyopathy, damage to the heart, leading to diastolic dysfunction and eventually heart failure. Macrovascular disease leads to cardiovascular disease, to which accelerated atherosclerosis is a contributor for: *i)* Coronary artery disease, leading to angina or myocardial infarction, *ii)* Stroke (mainly the ischemic type), *iii)* Peripheral vascular disease, which contributes to intermittent claudication as well as diabetic foot, and *iv)* Diabetic myonecrosis (1,2,12). DM is the fifth leading cause of death worldwide, claiming nearly 3 million lives annually (1,2). Direct patient costs for treating diabetes are estimated to range from $1,000 to $15,000 per year in Canada (7).

On a societal level, DM has significant implications on health care spending. Over 2 million Canadians live with diabetes, which accounts for an estimated cost of the health care to 15.6 billion dollars in 2010 and is expected to reach $19.2 billion by 2020 which 25% is attributed to direct, medical expenditure, whereas 75% attributed to loss of production due to morbidity and mortality associated with the disease (13). Therefore effective treatments to

prevent DM and its complications are critical to control the rising individual and societal costs associated with this disease.

1.1.4. Classification of DM

Two major classes of DM are differentiated by distinct pathophysiologic processes which lead to a common hyperglycemic symptom; Type 1 and 2. The term "Type 1 DM" has universally replaced several former terms, including: “childhood-onset diabetes”, “juvenile diabetes”, and “insulin-dependent diabetes mellitus (IDDM)” (2,14). Type 1 DM which typically occurs in childhood and adolescence of juvenile onset, is characterized by the absence of insulin production. Two subgroups of type 1 DM exist: The more prevalent Type1a DM, is an autoimmune disease resulting in pancreatic β cell destruction and complete absence of insulin secretion. Type 1b DM, in contrast, lacks the presence of autoimmune markers yet presents with destruction of insulin secreting β cells by an unknown mechanism (2,14). Likewise, the term "Type 2 DM" has replaced several former terms, including: “adult-onset diabetes”, “obesity-related diabetes”, and “non-insulin-dependent diabetes mellitus (NIDDM)” (2,14). Type 2 DM, on the other hand, can be caused by one or a combination of the following pathophysiologic processes: insulin resistance, impaired insulin secretion and/or increased glucose production leading to hyperglycemia. Type 2 DM most commonly presents during adulthood, however, the incidence of juvenile onset type 2 DM is increasing. Between 8-45% of new juvenile diabetics are being diagnosed as type 2 diabetics, depending on their location worldwide (15). The

risk of type 2 DM is increased with age, obesity, and sedentary lifestyle. Beyond these two types, there is no agreed-upon standard nomenclature. Various sources have defined “type 3 diabetes” as, among others, “gestational diabetes” which is another form of diabetes which occurs in pregnant females and is characterized by insulin resistance. This form of diabetes affects approximately 4% of pregnant females. “Insulin-resistant type 1 diabetes” or “double diabetes”, type 2 diabetes which has progressed to require injected insulin, and “latent autoimmune diabetes of adults” or “LADA” or "type 1.5" diabetes, and “maturity onset diabetes of the young” or “MODY” which is a group of several single gene (monogenic) disorders with strong family histories (2,14).

1.2. TYPE 1 DM

Type 1 Diabetes Mellitus (T1DM) has universally replaced several former terms, including: “childhood-onset diabetes”, “juvenile diabetes”, and “insulin-dependent diabetes mellitus (IDDM)”. Recent trends suggest that the age of onset of T1DM is decreasing and it is not exclusively a childhood problem; the adult incidence of T1DM is noteworthy, many adults who contract this disease are misdiagnosed with type 2 due to confusion on this point (1). T1DM is an autoimmune disease that results in specifically destruction of insulin-producing β cells of the pancreas. It is characterized by an absolute lack of insulin secretion and treatment therefore centers on the replacement of insulin (1,16-18). Lack of insulin causes an increase of fasting blood glucose (around 70-120 mg/dl in non-diabetic people) that begins to appear in the urine above the renal threshold (about

190-200 mg/dl in most people), thus connecting to the symptom by which the disease was identified such as glycosuria, polyuria, polydipsia, and polyphagia. These symptoms may develop quite rapidly (weeks or months) in type 1 diabetic patient, particularly in children. There is currently no clinically useful preventive measure against developing T1DM. Most people who develop T1DM were otherwise healthy and of a healthy weight on onset, but they can lose weight quickly (despite normal or even increased eating) and irreducible mental fatigue, if not promptly diagnosed (1,2). Although the cause of T1DM is still not fully understood, there is direct evidence of autoimmunity in the majority of patients with T1DM demonstrating that immunological, in addition to genetic and environmental factors play a role in the development of this disease. High glycemic lability, extreme sensitivity towards insulin, or hypoglycemic unawareness are the episodes which can be caused by large fluctuations of blood glucose levels as well as lack of adequate prodromic symptoms in the some severe cases of patients with T1DM. This can be extremely dangerous and concerning for patients as hypoglycemic episodes can lead to coma, seizures, or fatality (1,2).

1.2.1. Epidemiology and Etiology of T1DM

While T1DM comprises only 5-10% of patients with DM, an estimated 17 million people are afflicted with T1DM with an increasing incidence of ~3% each year in most countries (1,12,16). The fraction of T1DM in different parts of the world differs; this is likely due to differences in both the rate of T1DM and other

types of DM, most prominently type 2. These suggest strong evidence for the roles of genetic and environmental factors in the etiology of T1DM (1). It is believed that a combination of genetic, environmental, and immunologic factors contribute to the onset of the disease.

1.2.1.1 Genetic Factors

T1DM is a polygenic disease, meaning many different genes contribute to its expression. Depending on locus or combination of loci, it can be dominant, recessive, or somewhere in between. It has been demonstrated that the susceptibility to the development of T1DM is most significantly influenced by human leukocyte antigen (HLA) class II genes (19). The strongest gene, IDDM1, is located in the HLA II region on chromosome 6, at staining region 6p21. This is believed to be responsible for the histocompatibility disorder characteristic of T1DM meaning insulin-producing beta cells display improper antigens to T cells. This eventually leads to the production of antibodies that attack the beta cells (19). Weaker genes are also located on chromosomes 11 and 18. There have been several mechanisms put forth to explain the functional differences between various HLA class II molecules, and the reason of some haplotypes appear to be protective and others are associated with an increased risk of T1DM. While the HLA-DQ6 genotype appears to protect against the development of T1DM, two HLA class II haplotypes in particular HLA-DR3 and HLA-DR4 have been associated with an increased risk of developing T1DM. At least one of the HLA-DR3 or HLA-DR4 genotypes is present in 95% of Caucasian patients with T1DM

(2). Specifically, possession of the HLA-DRB1*03.DQ2 and HLA-DRB1*04.DQ8 predisposes individuals to T1DM; however the presence of these HLA risk haplotypes in no way guarantees the eventual onset of T1DM (20-23). 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 (17-19,24-26).

1.2.1.2. Environmental Factors

Environmental factors can strongly influence expression of T1DM. Monozygotic twin studies have demonstrated that type T1DM concordance ranges between 30 and 70% (1,2,12,16). This shows that environmental factors, in addition to genetic factors, influence disease prevalence. Enteroviral infections, breastfeeding, the early presence or absence of certain foods, birth weight,

childhood over-nutrition, maternal islet autoimmunity, and negative stressors have all been correlated with the prevalence of type 1 DM through observational studies (27-29). Many viral infections have been potentially identified in the etiology of T1DM, including, but not limited to, rubella, coxsackie, mumps, and cytomegalovirus (CMV) infections (27). Rubella infections that occur *in utero* typically result in the development of T1DM in 12-20% of individuals most likely by molecular mimicking of β cell antigens as presented to host 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: early exposure to cow's milk, exposure to nitrosamines, and exposure to wheat proteins. Early exposure to cow milk or lack of breast milk proteins has been the most extensively studied dietary contributor to T1DM (34-37). One of the leading hypotheses is that infants who were fed cow's milk instead of human's milk demonstrated an increase in the level of IgG antibodies during their first 9 months (38-40). This could be due to the incomplete induced oral tolerance to insulin as it found the human's milk contains approximately four times the amount of insulin compared to cow's milk (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. Therefore, foods which are high in nitrates and nitrites such as smoked meats are hypothesized to contribute to the onset of T1DM by damaging the β cells (43,44). Streptozotocin (STZ) which is used as an antibiotic and antineoplastic agent used in chemotherapy for pancreatic cancer is an N-nitroso compound and can induce diabetes in experimental animals by killing beta cells, resulting in loss of insulin production (43,44). Wheat proteins have been found to cause aberrant immune responses in susceptible individuals (i.e. Celiac's disease). 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 suggesting a partial association of wheat proteins in the etiology of T1DM (45-47). In the T1DM animal model, the BB rat, wheat gluten is a potent diabetic antigen (48,49).

1.2.1.3. Immunological Factors

The autoimmune response in T1DM is targeted towards specialized insulin producing β cells, which are clustered in the islets of Langerhans within the pancreas. It was hypothesized that an environmental trigger and an underlying genetic susceptibility initiates an autoimmune response, resulting in the progressive destruction of the β cells. The primary evidence for autoimmunity in T1DM includes insulitis and the presence of circulating autoantibodies. Beta

cell auto-antigens, dendritic cells, macrophages, T cells and B cells are known to be involved in the pathogenesis of autoimmune diabetes (50,51). Three major autoantigens have been identified namely: glutamic acid decarboxylase (GAD), insulin auto-antibodies (IAA), and certain islet tyrosine phosphatases (ICA512 or IAA and IA2β) (17,18,24). Beta 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. It is estimated that >90% of newly diagnosed patients with T1DM have one or more antibodies against the specific autoantigens mentioned above in comparison to the 3.5-4% of patients withnot the disease, however this later group is at greater risk of developing T1DM (52). Autoantibodies themselves do not seem to be the cause of the condition as adoptive transfer of these autoantibodies does not result in the disease state whereas transfer of T cells does (52). Animal models of T1DM have demonstrated that T cells play a critical role as effector cells which directly mediate β cell killing (50,51). 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 and cytotoxic T cells (T_C cells) are believed to destroy β cells by apoptosis via Fas-Fas ligand and by granzyme and perforin (50,51).

1.2.2. Pathophysiology of T1DM

In T1DM, pancreatic β cells in the islets of Langerhans are destroyed or damaged sufficiently to effectively abolish endogenous insulin production. This etiology distinguishes T1DM from T2DM. The islets of Langerhans are located in

scattered clusters within the pancreas and are the secretory component of the endocrine function of the pancreas (53,54). The islets are typically most concentrated in the body and tail of the pancreas. In humans, islet cells supply all of the pancreatic endocrine secretions, despite being only 1-2% of the total pancreatic mass. The islet cell is further comprised of four major well known cell types: *i)* α cells which secret glucagon. Glucagon stimulates the hydrolysis of glycogen by the liver. *ii)* β cells which produce insulin. Insulin, 5,800 kD globular protein 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. *iii)* δ cells which make somatostatin. Somatostatin dampens the effects of insulin and glucagon by suppressing the secretion of both insulin and glucagon. *iv)* Pancreatic polypeptide secreting cells (PP cells). Pancreatic polypeptide is expressed exclusively in the pancreas, however to date, its function remains unknown (1). In humans, the islet cell is composed of approximately 80% β cells which are arranged in a central core surrounded by a mantle of the other three cell types (53,55). It is widely speculated that only 10-30% of the endocrine cells are necessary for maintaining normoglycemia (56). Therefore the key to the pathophysiology of DM is insulin (57). Insulin is an essential anabolic hormone that promotes growth, regulates fuel mobilization and storage by promoting glycogen synthesis, protein synthesis, lipogenesis, and stimulating cell growth and differentiation (2,58), and is required for the achievement of metabolic homeostasis. In a normal individual, blood glucose levels 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.

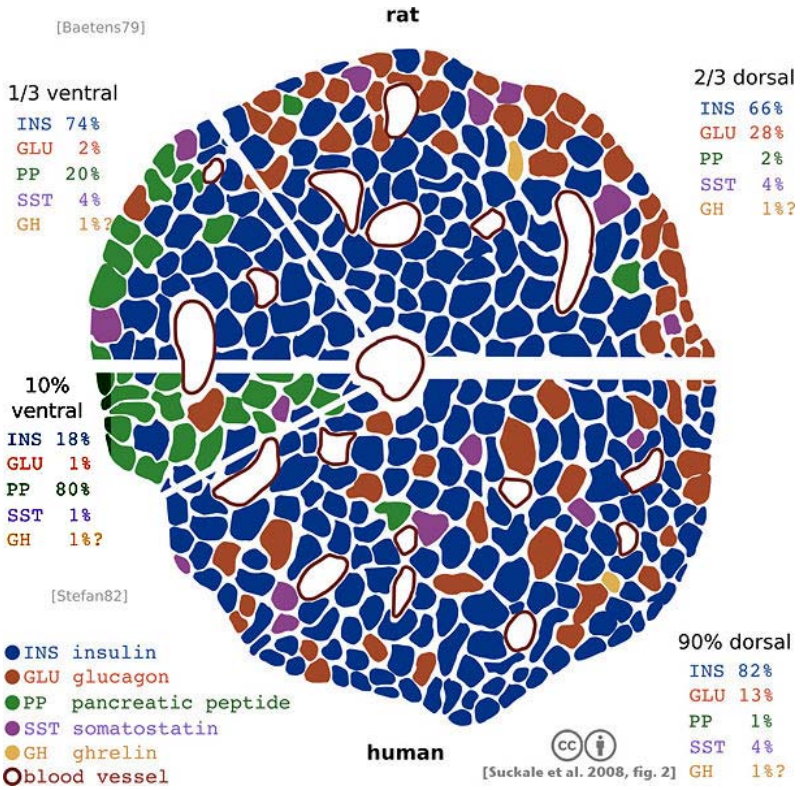

Figure 1.1: Schematic of cellular composition of rat and human pancreatic islets; shows fraction of dorsal and ventral pancreas as well as insulin, glucagon, PP (pancreatic polypeptide), somatostatin, and ghrelin cells (59).

In an individual with T1DM, 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 (1,2). A prolonged period 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 (60-62). In addition, the

increased demand on protein as an energy source can result in muscle wasting, during periods of prolonged insulin deficiency (63,64). Though the blood is replete with glucose, most of this glucose cannot be taken up by cells that require insulin dependent glucose uptake, resulting in urinary excretion of the carbohydrate (1,2).

1.2.3. Diagnosis and Clinical Features of T1DM

The diagnosis of T1DM, and many cases of T2DM, is usually prompted by recent-onset symptoms of polyuria, polydipsia, polyphagia, and often accompanied by weight loss (52). Patients may also initially present with diabetic ketoacidosis (DKA), an extreme state of metabolic dysregulation characterized by the smell of acetone on the patient's breath; nausea; vomiting and abdominal pain; and any of many altered states of consciousness or arousal (such as hostility and mania or, equally, confusion and lethargy). In severe DKA, coma may follow, progressing to death. The diagnosis of other types of diabetes is usually made in other ways. These include ordinary health screening; detection of hyperglycemia during other medical investigations; and secondary symptoms such as vision changes or unexplainable fatigue. DM is also diagnosed by: *i)* fasting plasma glucose level (defined as no caloric intake for eight hours prior to testing) at or above 126 mg/dL (7.0 mmol/l). *ii)* plasma glucose at or above 200 mg/dl (11.1 mmol/l) two hours after a 75 g oral glucose load as in a glucose tolerance test (52). While not used for diagnosis, an elevated level of glucose irreversibly bound to hemoglobin (termed glycosylated hemoglobin or HbA1c) of 6.0% or

higher (the 2003 revised U.S. standard) is considered abnormal by most laboratories; HbA1c is primarily used as a treatment-tracking test reflecting average blood glucose levels over the preceding 90 days (approximately) which is the average lifetime of red blood cells which contain hemoglobin in most patients to confirm pre-existing hyperglycemia (52). The current recommended goal for HbA1c in patients with diabetes is <7.0%, which is considered good glycemic control, although some guidelines are stricter (<6.5%). People with diabetes who have HbA1c levels within this range have a significantly lower incidence of complications from diabetes, including retinopathy and diabetic nephropathy (52). The most definite laboratory test to distinguish T1DM from T2DM is the C-peptide assay, which is a measure of endogenous insulin production since external insulin has not included C-peptide. During insulin biosynthesis, proinsulin is cleaved within secretory granules to insulin and C-peptide in a 1:1 ratio. This relationship is clinically significant in the quantification of insulin secretion. Insulin enters the portal venous system where approximately 50% is immediately metabolized by the liver (65). Insulin has a rapid plasma half-life and undergoes concentration dependent endocytosis and degradation in peripheral tissue making direct quantification. In contrast, C-peptide has a longer plasma half-life and is cleared by the kidneys in a concentration independent manner. As a result, quantification of insulin secretion can be accurately measured indirectly by plasma C-peptide levels. The presence of anti-islet antibodies, or lack of insulin resistance, determined by a glucose tolerance test, would also be suggestive of T1DM (2,57,65).

1.2.4. Treatment of T1DM

T1DM is lethal unless treated with insulin. Continuous glucose monitors have been developed and marketed which can alert patients to the presence of dangerously high or low blood sugar levels. Hypoglycemia is an acute complication of several diabetes treatments. It is rare otherwise, either in diabetic or non-diabetic patients. The patient may become agitated, sweaty, and have many symptoms of sympathetic activation of the autonomic nervous system resulting in feelings akin to dread and immobilized panic. Consciousness can be altered or even lost in extreme cases, leading to coma, seizures, or even brain damage and death. In patients with diabetes, this may be caused by several factors, such as too much or incorrectly timed insulin injection, too much or incorrectly timed exercise (exercise decreases insulin requirements) or not enough food (specifically glucose containing carbohydrates). The variety of interactions makes cause identification difficult in many instances (1,2). Several alternative approaches for delivering insulin in response to fluctuating glucose levels have been attempted with varied success which can be categorized to *i)* insulin replacement therapies and *ii)* β cell replacement therapies (1,2,66).

1.2.4.1. Insulin Replacement Therapy

Using the purified insulin generated by Collip, a biochemist from the University of Alberta, Banting and Best were able to demonstrate the effectiveness of insulin in treating patient of T1DM (67). Injection is the traditional and still the most common method for administering insulin; jet

injection, indwelling catheters, and inhaled insulin has also been available. In 1978, insulin became the first recombinant human protein to be manufactured (68) and today the most common types of insulin are biosynthetic products produced using genetic recombination techniques; formerly, due to the similar structure across species, bovine and porcine insulin have been extracted and used in the treatment of diabetes (57,67). Despite their similarities, occasionally complications were encountered from animal insulin, such as skin rashes (68). A combination of dietary and exercise modification and subcutaneous insulin delivery remains the standard therapy for patients with T1DM. Advances in insulin formulation continue to strive towards the optimal combination of basal and bolus administration to regulate blood glucose levels (69). Short-acting insulin formulations such as Novorapid, Humalog, and Aprida, are designed to be rapidly absorbed and coincide with post-prandial spikes in blood glucose levels, while long-acting insulin formulations such as Levemir and Lantus are aimed at controlling basal blood glucose levels for both bolus and maintenance requirements (70). New formulations, including inhaled insulin are designed to be rapidly absorbed and timed with meals (69). The safety and efficacy of inhaled insulin has been established (71) but clinical trials examining the ability to tightly control blood glucose levels using this delivery system are ongoing. An insulin pump provides a stable basal delivery of insulin; continuous subcutaneous insulin infusion is effective in decreasing the frequency of hypoglycaemic events (1), however, these devices are subject to potential malfunction and are more resource intensive than standard multiple injection insulin therapy (1). Microscopic or

nanotechnological approaches are under investigation as well, in one proposed case with implanted stores of insulin metered out by a rapid response valve sensitive to blood glucose levels. These are also in some sense, closed-loop integrated glucometer/insulin pump products, composed of three components: a glucose sensor, an insulin pump and a stable computer algorithm to control insulin delivery which could potentially increase the quality-of-life for some who have T1DM (66).

1.2.4.2. β Cell Replacement Therapy

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 (72). 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 (63). The major problem however was that intensive insulin therapy resulted in a three times higher incidence of hypoglycemic episodes, which is a major concern for patients as it can lead to coma, seizures, or death (73). This becomes even worse for these individuals with T1DM who already have an increased susceptibility for hypoglycemic episodes as well as patients who despite insulin therapy have inadequate glucose control. Thus, intensive insulin therapy is not suitable for all patients with T1DM, predisposing them for future complication. Due to the inability to control the development of secondary microvascular

complications with exogenous insulin therapy, more physiologic means of delivering insulin through β cell replacement via pancreatic or islet cell transplantation have been developed (73-76). 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 insulin producing β cells.

1.2.4.2.1. Whole pancreas and islet transplantation

Overall, while insulin therapy remains a life saving therapy for many patients with T1DM , a more physiological approach may be necessary in order to prevent future complications of diabetes for all patients suffering from the disease. In more extreme cases, a pancreas transplant can restore proper glucose regulation. However, the surgery and accompanying immunosuppression required is considered by many physicians to be more dangerous than continued insulin replacement therapy, and is therefore often used only as a last resort, such as when a kidney must also be transplanted, or in cases where the patient's blood glucose levels are extremely volatile. Given the favourable results seen in animal models (77,78), the first series of clinical whole pancreas transplant were performed at the University of Minnesota in 1966 (78). This initial series of whole pancreas transplantation were complicated by leakage of pancreatic exocrine secretions and transplant rejection, which continue to plague clinical pancreatic transplantation to this day (76,78). 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. Despite the morbidity associated with this major surgery, successful pancreatic engraftment results in euglycemia and normalization of glycosolated hemoglobin levels. 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 (79-82). 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 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 (79,83-86). Similar to any other type of 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 patients 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. In the current era, morbidity associated with pancreatic transplantation ranges from 7.0% to 11.5% (87). All-cause mortality in transplant wait-list patients receiving conventional treatment, is equivalent to that of patients receiving simultaneous pancreatic transplantation and renal transplantation for end-stage diabetic nephropathy, but significantly

worse for patients receiving pancreatic transplantation alone or after kidney transplantation (87). Due to its relatively high morbidity, pancreatic transplantation is primarily offered to uremic patients who are undergoing simultaneous renal transplantation (76). Technical difficulty and requirement for immunosuppressive drugs contributes to the unsatisfactory nature of pancreas transplant as a cure. Islet cell transplantation is expected to be less invasive than a pancreas transplant which is currently the most commonly used approach in brittle T1DM patients. In this procedure, islet cells are injected into the patient's liver, where they take up residence and begin to produce insulin. The liver is expected to be the most reasonable choice because it is more accessible than the pancreas, and islet cells seem to produce insulin well in that environment. The patient's body, however, will treat the new cells just as it would any other introduction of foreign tissue, unless a method is developed to produce them from the patient's own stem cells or there is an identical twin available who can donate stem cells. Because the immune system will attack the cells, patients now also need to undergo treatment involving immunosuppressants, which reduce immune system activity. Islet transplnatation will be discussed further in section 1.3.

1.2.4.2.2. Stem cell differentiation and gene therapy

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 (88). 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 (88). Several groups have reported successful differentiation of stem cells or precursor cells into insulin producing tissue (88-93). Two potential sources of stem cells exist, embryonic stem cells and mesenchymal stem cells. Embryonic stem cells (ESC) have the potential to differentiate into insulin producing β cells if guided along appropriate developmental pathways (94). A major hurdle in ESC research, however, are the ethical barriers surrounding ESC line procurement. Mesenchymal stem cells are derived from adult bone marrow, fat and several fetal tissues (95). Under appropriate environments, MSC are capable of differentiating into mesodermal, endodermal and ectodermal cells, including β cells. Several groups have demonstrated that nestin positive pancreatic ductal progenitor cells are capable of differentiating into insulin expressing cells both *in vitro* (96) and *in vivo* (97,98) with the ability to reverse hyperglycemia in diabetic mice (99). Stem cell research has also been suggested as a potential avenue for a cure since it may permit regrowth of islet cells which are genetically part of the treated individual, thus perhaps eliminating the need for immuno-suppressants (100). Gene therapy has been proposed as a treatment for T1DM. In order to evade β cell specific autoimmune attack, introduction of the insulin gene into non-β cells would theoretically result in subsequent autoimmune destruction of insulin producing

cells. Despite that fact that expression of biologically active transgenic insulin has been achieved (101), this strategy is currently limited by the inability to transfer to non- β cells complex glucose-sensing mechanisms required to secrete insulin within a narrow physiologic range (101).

1.2.4.2.3. 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 (102-105). Certainly a challenge in applying β cell regeneration to the treatment of patients with T1DM is the ongoing presence of autoimmunity. However, when autoimmunity is blocked, it has been demonstrated in humans (106-108) and rodents (109,110) that β cell function can partly recover. Using genetic lineage tracing, it has been demonstrated that regeneration of differentiated β cells, rather than differentiation of stem cells is the reason for β cell recovery (111). 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 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 (112). Recently however, several studies have succeeded in regenerating β cells in the animal model for T1DM, the Non-obese diabetic (NOD) mouse (113). 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 (114). 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 (115). The same combination of EGF and gastrin was also found to increase β cell mass in adult human pancreatic islets (115). Clearly this is an area of rapid growth; however it 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.

1.2.4.2.4. The bio-artificial pancreas

A biological approach to the artificial pancreas is to implant bioengineered tissue containing islet cells, which would secrete the amounts of insulin needed in response to sensed glucose. Encapsulation of the islet cells in a semi-permeable and protective coating has been developed to block the immune response to

transplanted cells, which relieves the burden of immunosuppression and benefits the longevity of the transplant (116). One concept of the bio-artificial pancreas uses encapsulated islet cells to build an "islet sheet" which can be surgically implanted to function as an artificial pancreas (116). This islet sheet design consists of *i)* an inner mesh of fibers to provide strength for the islet sheet; *ii)* islet cells, encapsulated to avoid triggering a proliferating immune response, adhered to the mesh fibers; *iii)* a semi-permeable protective layer around the sheet, to allow the diffusion of nutrients and secreted hormones; and *iv)* a protective coating, to prevent a foreign body response resulting in a fibrotic reaction which walls off the sheet and causes failure of the islet cells.

1.3. ISLET TRANSPLANTATION

With the improvements in islet isolation and the success seen in animal models, and compared with intensive insulin therapy, islet transplantation provided superior metabolic control, prevented hypoglycemic events and held the potential to decrease secondary complications of diabetes as well as required less invasive procedure and had lower risk of morbidity and mortality compared to whole pancreas transplantation (117,118).

1.3.1. History of Islet Transplnatation

In 1892 prior to the discovery of insulin, it was demonstrated by Von Mering and Minkowski that the pancreas was responsible for regulation of blood glucose as pancreatectomized dogs became hyperglycaemic (119). Minkowski

subsequently attempted to reverse diabetes in the diabetic dogs by autotransplanting fragments of the pancreas subcutaneously (120). In 1893, Watson-Williams and Harsant attempted to treat a young diabetic boy with subcutaneous implants of sheep pancreas fragments; while there was a temporary improvement in his glycosuria, the sheep pancreas xenograft was ultimately rejected and the boy died shortly thereafter (121). In 1964, Hellerstroem isolated islets for transplantation using a microdissection technique (122).The combined use of collagenase with mechanical force to disrupt the pancreas and liberate a greater proportion of islets for transplantation was first described by Moskalewski in 1965 (123) using chopped guinea pig pancreas. In 1967, Lacy and Kostianovsky used a method by distending the pancreatic duct prior to morcellation and collagenase digestion of rat pancreases (124). Using this technique, Lacy and Kostianovsky were able to isolate 300 islets from a single rat pancreas, demonstrating for the first time that an adequate number of viable islets could be isolated for transplantation. Lindall improved islet purity while preserving islet function by using differential density elutriation with Ficoll (125). In 1973, Scharp and colleagues refined the isolation of islets by substituting the use of a sucrose density gradient with a dialyzed Ficoll density gradient to separate unwanted acinar debris from rat islets (126). Ficoll, which was felt to provide a better osmotic environment for the islets compared to sucrose, varied in its ability to isolate viable islets from lot to lot. Scharp and colleagues also were able to demonstrate the ability to produce a high yield of viable rat islets for transplantation (126). Younoszai and colleagues performed the first attempt at

transplanting isolated rodent islets which resulted in a temporary amelioration of hyperglycemia in a diabetic rat model (127). The first sustained reversal of chemically induced diabetes in a rodent model, however, was demonstrated by Ballinger and Lacy in 1972 by transplanting 400-600 isolated rat islets either into the peritoneal cavity or in the thigh muscle of rats. They showed that when the islet isograft was removed from the thigh muscle site, the recipient rats returned to a hyperglycemic state (128). The introduction of intraductal infusion of collagenase which permits collagenase to more effectively digest surrounding connective tissue permitted a greater yield of isolated islets (129,130).

Several sites of transplantation have been investigated through the course of islet cell transplant development. Transplantation into intramuscular and intraperitoneal sites by Ballinger and Lacy (128) were successful in reversing diabetes, however, consistent results were difficult to achieve. Rechard and Barker later increased the number of rat islets transplanted to 800-1200 islets injected into the intraperitoneal cavity showing a complete reversal of diabetes for the first time (131). Numerous other sites of transplantation have been investigated with varied success including intraperitoneal (128,132-135), intraportal (132,134,136-139), intrasplenic (140-142), omental pouch (143-145), renal subcapsular (140-142,145-150), thymus (151,152), testicles (153), ventricles of the brain (154) and bone marrow (155,156). Under normal circumstances, insulin is delivered from the pancreas into the portal vein. Therefore, intraportal transplantation may provide a more physiologic site for delivery of insulin by lower number of transplanted islets. In 1973, Kemp and colleagues demonstrated

that intraportal transplantation of islets could successfully and consistently normalize hyperglycemia in a diabetic rat model (136). However there are major disadvantages with this site: *i)* severe procedural complication include bleeding, thrombosis, biliary puncture, and a transient increase in aminotransferase and arteriovenous fistula, *ii)* infusion into the portal vein also increased portal pressure (157), *iii)* islets are in direct contact with the blood in this site, making them more susceptible to IBMIR and subsequent losses in engrafted islet tissue (158), *iv)* intrahepatic islets may also display abnormalities due to exposure to high concentrations of glucagon, diabetogenic immunosuppressive drugs and their metabolites, as well as toxins from the gastrointestinal tract (159), *v)* biopsies are also difficult to acquire, therefore monitoring the graft becomes both difficult and more risky (159). Thus, clearly a more optimal site for implantation will have to be looked at in order to increase the safety and efficacy of this procedure. Small animal experiments demonstrated that the concept of β. cell replacement by transplantation of isolated endocrine islets was indeed possible and opened the door for experiments to come. One of the major barriers to human clinical trials was the limitation of adoption of techniques to isolate rodent islets to be used in human islet isolation. Further refinements in human islet isolation process followed in the subsequent years with the introduction of indraductal infusion of Liberase, the development of a semi-automated dissociation chamber (160), and the utilization of COBE for islet purification (118,161). These developments allowed for procurement of viable islets with increased purity, and enhanced the reproducibility and consistency of the human islet isolation procedure. These

combined improvements facilitated the large-scale isolation and clinical applicability of the procedure (118).

The first attempt at clinical islet transplantation occurred in 1977 and between 1977 and 1989 fifty-six cases of clinical islet transplantation were described with variable success in attaining transient C-peptide secretion (162). In 1989, Warnock and colleagues from the University of Alberta reported for the first time the ability of islets from human cadaveric donors to result in sustained C-peptide secretion after a synchronous intraportal islet and orthotopic kidney transplantation (162). Shortly thereafter, Lacy and colleagues at Washington University reported the ability of intraportally transplanted human cadaveric islets to result in insulin-independence in a type 1 diabetic patient (163). These landmark studies answered a key question by demonstrating the ability of human islet allografts to maintain normoglycemia in immunocompromised patients with T1DM. Consistent and sustained insulin independence remained elusive in clinical islet transplants that were to follow throughout the 1990's with only 12.4% of 267 islet transplant recipients sustaining insulin independence for 1 week and only 8.2% maintaining insulin-independence after 1 year (164). This was drastically changed in the year 2000 as a landmark study done by the Edmonton group demonstrated that seven out of seven patients were able to achieve and maintain normoglycemia for greater than one year post-transplantation by using a sufficient amount of fresh isolated islets and a glucocorticoid-free immunosuppressive regimen was used (164). This trial, later referred to as Edmonton Protocol, utilized a mean of 11, 546 islet equivalents per

kilogram of human patient's body mass, as well as an immunosuppressive protocol which included the use of sirolimus, tacrolimus, and anti-interleukin-2-receptor antibody therapy to protect against rejection (164). A five year follow-up study by the Edmonton group has performed over 140 islet transplantations in 85 patients. The patients that received islet transplantation revealed improved glucose stability and near prevention of hypoglycemic episodes. Although after five years follow-up 87% of patients maintained insulin-independence for 1 year, however, only 9% of patients remained insulin-independent despite the fact that 80% demonstrated some form of graft function through sustained C-peptide production and HbA1c levels were markedly improved (157,165,166).At present, over 500 patients have received islet transplantations at over 50 institutions worldwide in the past 5 years (167,168), with an international multicenter trial demonstrating its feasibility and reproducibility (169).

1.3.2. Barriers To Islet Transplantation

There are several barriers to the successful widespread implementation of islet transplantation for all T1DM patients. The major barriers are: shortage of donor pancreatic tissue, the immune mediated rejection of the transplant and the requirement for continuous administration of immunosuppressive drugs, and the potential recurrence of autoimmunity.

1.3.2.1. Shortage of Donor Tissue

As in almost all types of clinical transplantation, the number of potential recipients far out numbers the number of donors. 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 (170-174). 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) (158,175-178). Problems such as IBMIR, a thrombotic / inflammatory process, continue to be acknowledged as a major contributor toward the initial graft loss and subsequent engraftment. Briefly IBMIR happens when transplanted tissue comes in contact with host blood; inflammatory mediators (i.e. tissue factor and MCP-1) cause a rapid activation of the coagulation and complement systems, binding of platelets, and infiltration of polymononuclear granulocytes (179,180). This results in damaging of islets and thrombus formation which disrupts islet morphology and integrity. The clotting also prevents proper engraftment by preventing access to blood vessels (181). Different methods have been investigated to address this issue, including using low molecular weight dextran sulphate (176), heparin (179), compstatin (182), thrombin inhibitor (175), and other strategies that will prevent IBMIR such as genetic modification of donor in case of islet xenotransplnatation (158,183,184). One potential solution to the shortage of donor pancreatic tissue is the use of animal sources of insulin producing tissue, referred

to as xenotransplantation. Islet Xenotransplantation will be discussed further in section 1.4.

1.3.2.2. Immune Mediated Rejection of Transplanted Islet Grafts

To date all of the organ transplanted recipients require continuous administration of immune-suppressive drugs to prevent rejection process. Chronic administration of immunosuppressive drugs results in significant toxicity and harmful side effects, limiting the patient population for which this treatment is appropriate. Reported side effects of the immunosuppressive drugs used in clinical islet transplantation included mouth ulcers, ovarian cysts, diarrhea, acne, edema, anemia, and pneumonia (157,185). Thus, with the high level of side effects of the current anti-rejection therapies, islet transplantation remains limited to brittle patients with T1DM not all of the individuals with T1DM particularly children with this disease. 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.

1.3.2.3. 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 (103,186-188). Studies have shown that auto-reactive T cells persist in the body of the diabetic patient indefinitely (112). Despite this finding, success has been achieved in preventing the autoimmune

destruction of transplanted islet grafts in NOD mice. 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 (PLNC) became normoglycemic and tolerated minor antigen-disparate islet grafts for >100 days and syngeneic islet grafts indefinitely (189). 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 (190). Thus, in terms of graft rejection, it is often difficult to distinguish between immune mediated rejection and autoimmune mediated rejection.

1.4. PORCINE ISLET XENOTRANSPLANTATION

The goal of future strategies to treat T1DM is to provide an unlimited source of islets while avoiding the need for immunosuppression which is currently required in islet cell transplantation protocols. It appears that transplantation of xenogeneic sources of islets may provide a near-term solution to the shortage of islet donors. Historically, the first attempt at clinical islet xenotransplantation was undertaken by Watson-Williams and Harsant in 1893 by using pancreatic fragments from a sheep transplanted in a fifteen year old patient suffered from fatal ketoacidosis (118). Other sources of xenogeneic islet tissue for transplantation which have been investigated are: *i)* bovine islets (191-193) , *ii)* porcine islets (including fetal, neonatal, and adult) (194-196), and *iii)* fish Brockmann bodies (197-199). Pigs represent an attractive source of islets for at

least four reasons: First, pig and human islets are morphologically similar and pig insulin is structurally similar to human insulin (just one amino acid difference) and has been used clinically for the treatment of diabetes for years, demonstrating the efficacy of porcine insulin in reversing hyperglycemia in humans (200,201). Second, the supply of pigs is potentially unlimited because of shortened gestation and large litters (195). Third, genetically engineered pigs lacking antigens readily recognized by the human immune system could limit the extent of immunosuppression required to prevent rejection (183). Fourth, pigs can be housed in pathogen-free environments (202).

1.4.1. Age of Porcine Islet Donors

Several groups have investigated the use of porcine islets to experimentally correct diabetes in a variety of animal models. While there is a support for using islets of porcine origin, the optimal age of pigs from which islets should be harvested is still being debated. Three main age groups have been investigated including adult, neonatal, or fetal. Advantages and disadvantages of each donor source can be identified, however, to date successful large animal islet allotransplantation (143,203) and non-human primate islet xenotransplantation has only been achieved using neonatal and adult porcine islets (204-206).

1.4.1.1. Adult Porcine Islets

In 1974, Najarian and his colleagues from the University of Minnesota described the first successful attempt at isolating intact islets from adult pigs,

using a technique modified from Lacy's rodent islet isolations, involving duct dilation with cold Hank' s solution, collagenase digestion and ficoll gradient separation. They demonstrated the presence of circulating insulin following autograft and allograft transplantation in pancreatectomized pigs (207). In 1986, Lacy and his colleagues further refined the process of adult porcine islet isolation by dilating the pancreatic duct with collagenase solution prior to morcellation and ficoll gradient separation (160). Using this technique, a high yield of purified islets were obtained which demonstrated function *in vitro* by glucose stimulation insulin assay and transient *in vivo* function when transplanted into immunosuppressed B6 mice (160). Using a modification of the automated system for human islet isolation, Ricordi and colleagues were able to obtain an average of 255,000 islets per pancreas from 10 to 36 month old pigs with an islet purity of 85-90% (196). Advantages of the use of adult porcine tissue argue that: *i)* a larger number of islets can be obtained from adult pancreases, *ii)* the islets are mature at the time of isolation, are larger in size, and the potential for engraftment is better and is able to reverse hyperglycemia immediately in rodent and non-human primate models (196,204,206,208-213). However adult porcine islets are more fragile and difficult to maintain in culture for the purposes of decreasing immunogenicity or cryopreservation and are more susceptible to ischemic and hypoxic damages (196,208,214,215), *iii)* the quantity of islets that are isolated from an adult pancreas are dependent on the age, breed, and organ quality, as well as activity and lot of collagenase, all of which result in significant variability in islet yields (216-219), *iv)* are potentially more immunogenic furthering the need

for immunosuppression (204,220), *v)* logistically, maintaining pigs in pathogen-free environments long-term becomes both difficult and costly, greatly limiting the applicability of this treatment (202,216), and lastly *vi)* it would become difficult to ensure pigs of a proper age are available for transplantation if you need to wait long periods of time before harvesting their pancreas (202,216).

1.4.1.2. Neonatal Porcine Islets

In 1996, Korbutt and colleagues translated the simplified procedure used for fetal porcine islet isolation to isolate neonatal porcine islets (NPI) from 1-3 day old pigs with the goal of attaining a more functionally mature islet source (195). NPI were demonstrated to secrete insulin upon high-concentration glucose stimulation *in vitro* and transplantation of 2,000 NPI aggregates under the kidney capsule of an immune-deficient mouse was sufficient to reverse alloxan-induced diabetes (195). However, while postnatal maturation of NPI results in immediate *in vitro* insulin secretion, transplantation of NPI into immune-deficient mice results in delayed reversal of hyperglycemia at 8 weeks post-transplantation (195). Numerous studies have shown that NPI are capable of restoring normoglycemia in small and large animal models (143,144,195,196,203,205,214,221-227). It was shown that that Rhesus macaques that were transplanted and treated with a CD28-CD154 costimulation blockade regimen achieved sustained insulin independence for a median survival of >140 days (205). This study was the first to demonstrate that NPI are able to restore normoglycemia in non-human primates (205). A clinical trial of NPI and Sertoli

cell co-transplantation has also been performed in adolescent patients in Mexico, however, concerns were raised by the international community regarding the selection criteria of these transplant recipients as well as the results of this trial (228). 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 (195). Second, neonatal islets maintain considerable growth capacity, and may increase in number after subsequent transplantation (143). 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 (96,195,229). Fourth NPI are less susceptible to damage during culture and cryopreservation and are more resistance to hypoxia (214,215,225). Lastly neonatal pigs would require fewer facilities and resources to house and maintain prior to transplantation than adult pigs, particularly considering pathogen free facilities which would require months to mature (202). Some disadvantages associated with neonatal pancreatic tissue include: *i)* the inability of immature islets to immediately reverse hyperglycemia when transplanted as compared with mature islets, *ii)* the presence of potential antigens on the surface of the islets (i.e Galα(1,3)Gal) which could elicit an immune response by the recipient (214,222,230,231), and *iii)* the high number of donor piglets required for

transplantation and the limitations (i.e. labor-intensive nature of our current method) involved in preparing these cells for transplantation.

1.4.1.3. Fetal Porcine Islets

A simplified protocol to isolate porcine fetal islet-like cell clusters (FICC) was described by Korsgren and colleagues in 1988 (232). This isolation protocol, based on a protocol developed by this same group to isolate human FICC (233), aims to digest the pancreas into small clusters using collagenase and omits the steps of ductal infusion of collagenase and ficoll gradient separation of islet cells . To separate endocrine and exocrine components of the isolate, FICC are cultured for 7 days with every other day media changes (232). While it was noted that insulin secretion was quite uniform, the functional ability of fetal porcine islets was quite immature and stimulation of islets with glucose and theophylline yielded only minimal amounts of insulin release. Fetal porcine islets can be maintained in culture for up to 30 days (234) and during this time fetal porcine islets mature resulting in increased insulin secretion (232). Because FICC are functionally immature, they require up to 8 weeks to exhibit function in immune-deficient mice (194). In 1994, the Uppsala group transplanted porcine FICC intraportally into 10 insulin-independent diabetic renal transplant patients (235). While small amounts of porcine C-peptide were detected in the urine of these patients at 200-400 days post-transplantation, none of these patients attained insulin independence. 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. Like NPI, fetal porcine islets also express Galα(1,3)Gal, which could potentially mediate hyperacute rejection once transplanted (236-238). The major limitation with fetal islets however, is that they consistently demonstrate low insulin secretary response to glucose in both *in vitro* and *in vivo* and take months to correct hyperglycemia in animal models (194). Even more, relatively lower numbers of islets can be isolated from a single fetal pancreas compare to neonatal pancreas, necessitating a greater quantity of fetal pig donors in order to isolate sufficient quantities of islets for transplanting a single recipient. For example, in the study mentioned above approximately 100 pig fetuses were required to transplant each patient.

1.4.2. Porcine Islet Xenograft Rejection

The second major obstacles limiting clinical application of porcine islet transplantation is finding a safe and effective way to evade host immunity and the potent cellular response mounted against xenogeneic tissue. This barrier is of immense importance as further usage of harmful immunosuppressive therapies would only further limit the applicability of islet transplantation. Thus, safe and effective ways to achieve xenograft protection must be discovered in spite of the potent immune response. At present many experimental models involving porcine tissue utilize mice as recipients, which represents a discordant relationship (239). 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

(240,241). Pigs and humans are more closely related than pigs and mice (242), consequently the type of immune response which is elicited in each situation is different. 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 transplants (243,244). Also 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 (245). 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 (246). 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. The process of porcine islet xenograft rejection comprises of IBMIR, as well as innate and antibody responses and cell mediated immune responses.

1.4.2.1. Instant Blood-Mediated Inflammatory Reaction

Similar to human islets, porcine islet xenograft preparations are susceptible to instant blood-mediated inflammatory reaction (IBMIR) when exposed to both human blood *in vitro* and also when transplanted intravascularly in non-human primates (247). Without specific treatment to prevent IBMIR, porcine islets may be rejected within days post-transplantation and may explain

the acute rejection seen in some intraportal porcine transplant models (248,249), however, despite concomitant heparin therapy during intraportal infusion of porcine islets between 22-73% of islets are damaged at 24 hours post-transplantation (209). A potential reason for IBMIR is the presence of preformed complement binding antibodies to α- galactosyl alpha (1–3) galactose (αGal), a carbohydrate moiety found on many mammalian cells. Komoda and colleagues have developed a transgenic pig that overexpresses Nacetyl glucosaminyl transferase- III (GnT-III), an enzyme which competitively glycosylates trans-golgi carbohydrates and prevents the formation of αGal (208). When adult porcine islets from this transgenic model were transplanted into streptozotocin-induced diabetic monkeys, it was found that islets from the GnT-III transgenic pig did not undergo hyperacute rejection and showed less humoral and cellular rejection (208). While nonhuman primate studies demonstrating the successful long-term survival of both NPI and adult porcine islets suggest that both αGal and non-αGal mediated IBMIR can be overcome (204-206), the need for extraordinary volumes of islets for transplantation (25,000-50,000 IE/kg) suggests the need to consider the use of transgenic αGal-deficient pigs and/or the development of further therapies to prevent IBMIR. However, islet xenograft destruction by activation of the complement cascade is not the sole means of IBMIR as depletion of complement components by cobra venom factor significantly reduces porcine islet xenograft destruction but is not sufficient to completely prevent IBMIR (250).

1.4.2.2. Innate Immune Mediated Islet Xenograft Rejection

The role of innate immune cells in the rejection of porcine islet xenografts at current time is incompletely defined. Examining the immune reaction associated with fetal porcine islet xenografts in murine models, numerous types of innate immune cells have been temporally associated with rejecting xenografts including macrophage, NK cells and eosinophils (251-253). While eosinophils are temporally associated with fetal porcine islet xenograft rejection, in the absence of eosinophils islet xenograft rejection kinetics are unaffected suggesting a negligible role of these innate immune cells in the rejection process (252). Several studies have demonstrated that macrophage depletion results in prolongation of porcine islet xenograft survival when transplanted under the renal capsule (253), intraportally (254) and also intraperitoneal transplantation of microencapsulated islets (255). It appears that macrophage can act as effector cell that carries out porcine islet rejection. Yi and colleagues demonstrated that if $CD4^+$ T cell-activated macrophage were transferred to NOD-SCID mice, they were capable of rejecting fetal porcine islet xenografts (256). In contrast, when unactivated macrophages were transferred in this model, islet xenografts were not rejected (256). The role of NK cells in islet xenograft rejection, on the other hand is less clear. However, after administering anti-NK 1.1 mAb in a discordant adult porcine islet xenotransplant model, Wu and colleagues did not find a significant prolongation in porcine graft survival in mice (253).

1.4.2.3. T Cell Mediated Rejection

In a transplantation setting, two pathways of antigen recognition by T cells are capable to be activated. One, the direct pathway, wherein the host T cell recognizes antigen presented on the surface of donor antigen presenting cells APC (214). In the second pathway, the indirect pathway, the T cell is activated by recognition of antigen that is presented by host APC (214). These two pathways of antigen recognition are both capable of T cell activation and lead to an immune response, which results in the destruction of the transplanted islet graft. It has been well established that the indirect pathway of antigen recognition dominates when the phylogenetic disparity between the donor and recipient increase and that the direct pathway is increasingly active in the rejection of allogeneic transplants (243,244). The relatively disparate combination of porcine islets transplanted into mouse recipients, meanwhile, is completely dependent on the indirect pathway as MHC class II – deficient recipient mice are unable to reject their porcine islet xenografts indefinitely (244). Several studies in small-animal models using non-depleting anti-CD4 monoclonal antibodies (257), depleting anti-CD4 monoclonal antibodies (258) and $CD4^+$ T cell knock-out mice (259) have demonstrated the importance of $CD4^+$ T cells in this rejection process. These findings demonstrate that $CD4^+$ T cell activation by the indirect pathway is absolutely essential in porcine islet xenograft rejection in mouse animal models and consequently anti-rejection therapies to date have focused on preventing this interaction from occurring. While $CD4^+$ T cells play a central role in porcine islet xenograft rejection, $CD8^+$ T cells may play a minor role in rejection in the presence of $CD4^+$

T cells (244). These results provided further evidence that porcine islet xenograft rejection relies predominantly on $CD4^+$ T cells while $CD8^+$ T cells are only minimally involved in xenograft rejection (244,260,261). Thus, as T cells particularly $CD4^+$ T cells are mandatory for discordant islet xenograft rejection, therapies that specifically target this cell type/pathway without producing toxic side effects will need to be found. New ways of preventing the T cell mediated graft rejection are being investigated and will be discussed further in section 1.5.

1.4.2.4. Antibody Mediated Rejection

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. Antibody mediated hyperacute rejection of islet xenografts may occur by two possible mechanisms: complement mediated rejection and innate cell mediated rejection. 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 (262,263). 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 NPI 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 (230). While the expression of Galα(1,3)Gal on porcine tissue represents a significant concern, a recent study involving the transplantation of neonatal 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, this rejection was not associated with an increase in Galα(1,3)Gal antibody titres (205). Strategies to prevent humoral mediated rejection typically target T cells as T_H cells are required for the activation of B cells. Newer approaches are also under development which target co-stimulatory molecules present on B cells (264).

1.4.2.5. Autoimmune Recognition of Islet Xenografts

Given that the primary application of islet transplantation will ultimately be in patients with autoimmune diabetes, an important additional issue will be whether xenogeneic islets can be recognized by the pre-existing islet-specific autoreactive immune cells (261,265). There has been some indication that the autoimmune repertoire may be at least partially species specific. This is an important issue to resolve because the application of xenotransplantation, although attempting to solve the donor shortage, could have added biological benefit by introducing a tissue source that is relatively impervious to autoimmune recognition (261). This concept would predict that islet xenografts may enjoy preferential survival relative to allograft in some cases when grafted to autoimmune recipients (261). This property of enhanced islet xenograft survival has been shown in autoimmune diabetic NOD mice treated with anti-CD4 therapy (246,261). Such studies have been interpreted to mean that the autoimmune repertoire is relatively species specific (265).

1.4.3. Clinical Application of Porcine Islet Xenotransplantation

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

autoimmune diabetic human patient (118,168,266). Since that time, however, clinically applicable porcine islet xenotransplant models have been performed primarily in non-human primates. Despite the vast resources and expenses necessary, there is great interest in non-human primate models of porcine islet xenotransplantation as they likely represent a closer physiologic environment to that seen in humans. Soderlund and colleagues demonstrated that fetal porcine islets transplanted under the renal sub-capsule of cynomolgus monkeys underwent acute cellular rejection (267). When immunosuppressed with cyclosporine and 15-deoxyspergualin (DSG), cellular infiltration was delayed but ultimately the graft underwent rejection by day 15. This model confirmed that similar to small animal models, porcine islet xenotransplantation in primates also undergo cellular rejection. Nevertheless, focus has shifted to models that utilize portal vein injection of islets to more closely approximate the clinical model of islet transplantation. Portal vein transplantation models in non-human primates demonstrated that porcine islet xenotransplantation was feasible but were also acutely rejected despite the use of powerful immunosuppressant regimes (248,249). Both studies, however, lacked specific treatment to guard against IBMIR and likely reflect a rejection mechanism not seen in small animal models. Despite the fact that xenografts were rejected acutely after only 1-2 days, they did transiently result in detectable C-peptide levels (248,249). In 2004, Hering and colleagues successfully transplanted adult porcine islets intraportally into rhesus macaques and were able to achieve euglycemia (209). When injected intraportally, these transplants were not subjected to hyperacute rejection, putting

into question the role of αGal mediated hyperacute rejection. Beginning at 24 hours posttransplantation, however, these xenografts displayed evidence of cellular infiltration and subsequently were rejected with the infiltrate characterized by the presence of $CD4^+$ T cells, $CD8^+$ T cells and macrophage. The first published porcine islet xenotransplant trials were performed by Groth and colleagues in 1990 (235). Fetal porcine islets were transplanted in 10 insulin dependent diabetics who had previously or were undergoing renal transplantation. The fetal porcine islets were transplanted either via intraportal injection or under the kidney capsule. This pilot study demonstrated that islet grafts injected neither intraportally nor under the renal capsule resulted in immediate complications. Four of 10 patients did excrete porcine C-peptide several months post-transplant suggesting that hyperacute reaction did not occur and that survival of porcine islet xenografts is possible in human recipients. More recently, another human clinical trial was performed in Mexico by Valdes and his colleagues (228). Using a vascularized collagen sheath implanted subcutaneously, adolescents patients with T1DM were co-transplanted with NPI and Sertoli cells. Without immunosuppression it was reported that 50% of the transplanted patients saw a significant reduction in their insulin requirements and 2 of the 12 transplanted patients were transiently free of insulin injections. Given the fact that both NPI and adult porcine islets have been demonstrated to function in preclinical non-human primate models (204-206), it has been suggested that these sources will serve as the donor sources for first iteration islet xenotransplantation clinical trials (268). Increased regulatory controls on materials involved in direct human

exposure will likely play a large role in selection of a porcine islet source for transplantation (269). Close control of porcine islet donor herds will be mandated by regulatory boards involved in clinical trials of porcine islet xenotransplantation and will involve donor animals being designated pathogen-free (269). Breeding colonies can be established by early weaning or delivery by caesarian derivation into a pathogen-free housing facility and subsequent testing for pathogen free status. Current recommendations are for animals to be housed for 2 generations prior to being released as sources for clinical transplantation. Once born, islet donors will be maintained in pathogen-free conditions in compliance with Good Manufacturing Practice (GMP), fed pathogen free food and water and thoroughly some tested for bacterial, parasitic and viral pathogens (269). Therefore, while utilization of NPI as an islet donor source would necessitate the use of large numbers of islet donors, only breeding pairs would be maintained under GMP conditions while donor animals would be processed for islet transplantation soon after birth. On the other hand, while only 6 adult porcine islet donors would be required for a single human islet recipient, all 6 donor animals would need to be housed and tested under GMP conditions for 10 to 36 months prior transplantation which may escalate costs. Regardless of the source of porcine islet donor chosen, if clinical islet xenotransplantation is to succeed, automated means of isolating porcine islets will need to be developed to provide adequate donor islets to meet the volume of diabetic patients around the world.

1.4.4. Xenozoonosis

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 (270). In the past decade concern over latent, endogenous infectious agents has been raised in the use of porcine tissue for xenotransplantation. In particular, Porcine Endogenous Retrovirus (PERV) is a C-type retrovirus which is incorporated into the porcine genome. Three subtypes of PERV exist, classified based on the *env* subfamily expressed within the virus. PERV A and B is found in the genome of all pigs while some strains of pigs lack PERV C (271). There are currently three accepted approaches for detecting PERV infectivity, *i)*detection of PERV DNA sequences via PCR, *ii)* detection of PERV RNA sequences via RT-PCR and *iii)* immunologic assays detecting anti-viral antibody production using western blot analysis or ELISA (271). Detection of PERV DNA sequences is confounded by the presence of endogenous PERV sequences within porcine tissue samples. The use of real-time PCR improves sensitivity of this approach as PERV DNA sequences can be measured against porcine mitochondrial DNA sequences to rule out the presence of porcine tissue within a sample (271). Alternatively, RT-PCR may be used to detect transcribed PERV RNA sequences found in cells, infected tissue or packaged into viruses. However, expression of PERV RNA does not necessarily signal the release of viral particles as the majority of PERV loci within the pig genome contain frameshift mutations and stop codons preventing full transcription of the entire viral sequence (271). Lastly, detection of

anti-viral antibody production with various immunologic approaches is the most sensitive method for detecting ongoing PERV replication and infectivity. However, major drawbacks with immunological approaches are that other retroviral infections may result in false-positive tests and any anti-rejection therapies which downregulate B cell responses may also blunt the anti-viral antibody response (271). Evidence of PERV infecting human cells *in vitro* was first reported in 1997 (272). While it is not known what triggers expression of the retrovirus, *in vitro* (273) and *in vivo* (274,275) work suggests that mitogenic and post-operative hypoxic stress may trigger the expression of PERV. Despite experimental data to suggest the possibility of PERV transmission to xenotransplant recipients, retrospective reviews of human patients exposed to porcine tissue (276) and specifically porcine islets (277,278) have not identified cases of transplant recipients infected long-term with PERV. While current evidence suggests that PERV infection does not occur *in vivo* in human host cells, in this era of concern regarding zoonotic infections such as the avian flu and Creutzfeldt- Jakob disease, sensitive yet accurate testing for PERV is paramount as both true-positive and false-positive results of PERV infection *in vivo* would have a crippling effect on efforts at porcine islet xenotransplantation.

1.5. STRATEGIES FOR PREVENTING THE IMMUNE REJECTION OF TRANSPLANTED ISLETS

The goal to prevent islet graft rejection is to use of novel strategies or immunosuppressive and inflammatory blockade agents which can make

significant improvements to the outcome of clinical islet transplant program without having harmful side effects. These strategies and agents for preventing rejection of islet grafts should share four features in that they *i)* are non-diabetogenic or reduce the need for more diabetogenic immunosuppressive agents, *ii)* reduce initial damage of islet cells and promote engraftment, *iii)* induce tolerance, and *iv)* aim to manage the underlying autoimmune nature of T1DM in addition to stopping allograft rejection processes.

1.5.1. Immunosuppressive Drugs

There are many classes of anti-inflammatory and immunosuppressive drugs including: corticosteroids, calcineurin inhibitors, anti-metabolites and the mammalian target of rapamycin (mTOR) inhibitors. The majority of immunosuppressive drugs act in the induction phase of an immune response and inhibit the activation and/or proliferation of lymphocytes. Prior to the Edmonton protocol, immunosuppression of islet transplant recipients was governed by what was felt to be optimal therapy to sustain the existing solid organ graft. In that era, a combination of cyclosporine , corticosteroids, and azathioprine was administered to islet–kidney transplant recipients and to a limited number of islet-alone transplant recipients (279). 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 (280-282). In the 1980s it was reported that cyclosporine induced insulin resistance (283), and later it was revealed that cyclosporine was actually diabetogenic due to its toxicity towards β cells. At present cyclosporine is rarely implemented in transplantation mainly due to its deleterious side effects the most serious of which is nephrotoxicity (284). Corticosteroids have a wide-spread immunosuppressive impact and are also particularly diabetogenic (285). The β cell toxic effects of cyclosporine and corticosteroids led many clinical islet programmes to pursue steroid-free immunosuppression for islet transplantation (286-288). At present, patients which undergo islet transplantation are placed on a novel immunosuppressive regimen which includes the combination of sirolimus (rapamycin) and tacrolimus, along with anti-CD25 mAb induction, which improved outcomes of clinical islet transplantation (289). Rapamycin is an anti-fungal metabolite produced by the bacteria *Streptomyces hygroscopicus* (290,291). The target of rapamycin has been identified and incidentally, in mammals is referred to as the mammalian target of rapamycin (mTOR) (292). 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 (290,292,293). 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 (290). 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 (290). Tacrolimus, a macrolide antibiotic, is another type of calcineurin inhibitor. Tacrolimus (FK506) has a similar structure as rapamycin and 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. Tacrolimus is active at lower concentrations than cyclosporine, allowing physicians to administer the immunosuppressant with fewer associated side effects (290).

1.5.2. Monoclonal Antibodies (mAbs)

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 APC (294). The second signal is an antigen nonspecific signal triggered by the interaction of a pair of cell surface costimulatory molecules expressed on the T cell and APC which is termed Signal 2. This signal augments and amplifies activation and this is accomplished via decreasing the threshold of activation, increasing expression of adhesion molecules, and preventing anergy. Overall, this process leads to the production of cytokines, as well as proliferation and differentiation of effector cells which leads to a full blown immune reaction (295,296). 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 (295,297,298). It is now commonly accepted that full T cell activation and the type of response requires additional interaction of trafficking and costimulatory molecules present on the surfaces of T cells and APC (299). Targeting T cell activation pathways or blocking of these additional signals by targeting costimulatory and adhesion molecules using mAbs prevents complete activation of T cells with the specificity of their targets and their propensity for fewer side effects, then represents an attractive therapeutic target of anti-rejection therapy (299-305).

1.5.2.1. T Cell-Depleting mAbs

T-cell-depleting agents, such as alemtuzumab (Campath-1H; anti-CD52 mAb), hOKT3γ1 (Ala-Ala, anti-CD3 mAb), anti-T-cell globulin (polyclonal antibody) and diptheria immunotoxin anti-CD3, are currently being investigated in primates and plans are progressing to evaluate these in human islet transplantation. Alemtuzumab is an anti-CD52 antibody found to deplete lymphocytes and prevent T-cell activation through the CD45 pathway. Alemtuzumab is effective in the management of autoimmune diseases, including acute vasculitides and multiple sclerosis, and has been shown to be effective as an induction agent for renal transplantation when used along side either low dose cyclosporine (306) or sirolimus (307,308). An alternative strategy to depleting T-cells is the use of an Fc-receptor non-binding humanized anti-CD3 mAb

(hOKT3γ1, Ala-Ala), which has been used clinically in the single donor islet transplantation protocol in Minnesota with promising preliminary results (309).

1.5.2.2. T Cell Co-Stimulatory Receptor-Blocking mAbs

The discovery that TCR stimulation without co-stimulation can induce anergy (310) has led to intensive evaluation of co-stimulatory blockade in the transplantation field. Targeting of T cells with mAb has represented the focus of many anti-rejection regimens. Two strategies of steric interference between costimulatory receptors and their ligands have been proposed: *i)* a non-depleting mAb to block receptors or *ii)* receptor–Ig fusion proteins, which bind co-stimulatory ligands thereby precluding their binding to cognate co-stimulatory receptors on the T-cell. Many of the mAbs utilized in experimental models have not yet been applied clinically in islet transplantation; however mAbs to the IL-2 receptor form part of the foundation of the anti-rejection regimen of the widely successful Edmonton Protocol (164). 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 (311). Combining antibodies to the IL-2 receptor with calcineurin inhibitors has resulted in greater graft survival in many types of transplantation (312-315), because of the ability of these anti-rejection therapies to act synergistically. Several compounds that specifically target T-cell co-stimulatory molecules, including the CD28 and CD40L (CD154) pathways, have been explored in islet transplantation.

Targeting of B7 co-stimulatory molecules with a soluble fusion protein, CTLA4 (cytotoxic T-lymphocyte attenuator protein 4)-Ig was effective at preventing human islet graft rejection when transplanted into mice (316). 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 (317). Overall however, the above information shows that targeting this receptor-ligand pair or pathway can greatly reduce the immune responses involved in graft rejection and may possibly lead to tolerance induction via blockade of imperative co-stimulatory signals. A new wave of interest in co-stimulation blockade has emerged from the fusion protein CTLA4Ig and its even more potent analogue LEA29Y (Belatacept) (318). These fusion proteins bind to CD80 and CD86, blocking their interaction with the co-stimulatory receptor CD28 on T-cells. Results of a Phase III clinical trial in renal transplantation show reduced chronic allograft nephropathy in patients treated with LEA29Y when compared with cyclosporine (319).

1.5.2.2.1. Anti-CD154 mAb

CD40 is a transmembrane glycoprotein expressed on all APC (B-cells, dendritic cells, macrophages, among others), on activated T-cells, hematopoeic progenitor cells, as well as non-hematopoeic cells such as endothelial cells (320). Its ligand, CD154 (CD40L) is found on activated T and B cells, activated platelets (321), and on a number of other cells during inflammation such as peripheral blood mononuclear cells (322,323). CD40/CD154 interaction seems to provide

signals to both APC and T-cells directly. Binding to CD40 is critical for the maturation of APC, promoting the increased expression of co-stimulatory molecules (i.e. B7.1 and B7.2), adhesion molecules such as CD54 (ICAM-1), and antigen expression (320,324,325). Even more, APC have been shown to increase cytokine and chemokine production after CD40/CD154 interaction (320,325). For example, dendritic cells have been shown to secrete increased levels of TNF-alpha, IL-12, IL-8, all of which are known to be pro-inflammatory (326). The binding of CD40 has been shown to be extremely important in B cell differentiation and maturation and a fundamental role in class switching of antibody isotypes (323). The targeting of CD154 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 Programmed Death 1 (PD-1) stimulation was successful at inducing long-term survival of MHC mismatched islet allograft (327). Targeting of CD154 in xenogeneic islet transplantation appears to be equally as efficacious (328). Treatment with (CTLA-4) is effective in preventing the rejection of NPI transplanted into CD154 knockout mice (329), and additionally anti-CD154 monotherapy was effective at inducing tolerance to human islets transplanted into mice (248). Treatment with anti-CD154 and CTLA-4 was also effective at inducing indefinite rat islet xenograft survival in mice (330). Initial studies of an anti-CD40L mAb in non-human primates promised long-term allograft survival including in islet transplantation (331,332). Most recently it has be shown that the addition of anti-CD154 mAb to the established immunosuppressive regimes effectively prolonged neonatal and adult

porcine islet xenotransplantation in non-human primates (204-206). However, clinical trials in islet transplantation were not pursued following Phase I trials which revealed unanticipated microthrombotic events and the death of one subject (295,333,334). It has been suggested that the thromboembolic complication, which is also seen in non-human primates treated with humanized anti-CD40L, results from platelet activation and aggregation rather than a response to T-cell co-stimulation blockade (295,333,334). Administration of heparin during this therapy did reduce thromboembolic events (334). Anti-CD154 mAb has also been shown to be partially effective in autoimmune transplant models. DR-BB autoimmune diabetic mice treated with hamster anti-rat CD154 mAb significantly prolonged graft survival with no recurrence of diabetes for up to 461 days post-transplant (335). Allotransplantation of C57BL/6 mouse islets into spontaneously diabetic NOD female mice was significantly prolonged from a median 10 days with isotype control to 46 days with higher dose of anti-CD154 mAb (336).

1.5.2.3. Lymphocyte Trafficking Blockade mAbs

An alternative approach to traditional immunosuppression, which has generally targeted lymphocyte activation, is to inhibit lymphocyte migration to their site of activation. It is now well understood that lymphocyte activation and effector responses occur in distinct anatomical compartments, the migration to which is controlled by chemokines (337). Inhibitors of lymphocyte trafficking have been gaining popularity as immunomodulatory agents. Emerging compounds of interest include FTY720, a potent inhibitor of lymphocyte exit

from the thymus and lymph nodes, which depends on several chemokine systems (338). FTY720 is a non-specific antitrafficking agent; newer agents which specifically target one chemokine receptor subsystem either in the form of antibody blockade of chemokine receptors or small molecule chemokine receptor antagonists are currently being tested in preclinical models of islet transplantation (339,340). FTY720 has been investigated in primate models of islet transplantation with promising results in terms of safety and efficacy when combined with basiliximab and everolimus (a corticosteroid- and calcineurin-inhibitor-free immunosuppressive regimen) (341).

1.5.2.3.1. Anti-LFA-1 mAb

Leukocyte function associated antigen-1 (LFA-1, CD11a/CD18) is a member of a family of structurally and functionally similar leukocyte differentiation antigens (342,343) and was first identified in mice in 1981 (344). LFA-1 is widely expressed on hematopoietic cells including: T and B lymphocytes, natural killer cells, monocytes, macrophages and granulocytes (342,343,345-347). Its primary targets are ICAM-1 and ICAM-2 on endothelial cells, ICAM-1 and ICAM-3 on APC, and JAM-1 found at tight junctions of endothelial and epithelial cells (348,349). There are potentially important roles of this integrin which make it an attractive therapeutic target for evading immune rejection. 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 (350). 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 (351-357). Therefore, antibodies to LFA-1 are also effective at preventing graft rejection, because they can disrupt lymphocyte homing to site of a transplanted graft (356). 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 (358-360). It has also been reported that LFA-1 engagement through this signalling pathway can polarize the T cell towards a T_H1 phenotype (359). 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 T cells (361). Thirdly, LFA-1 plays a critical role at the interface between APC and T cells (361). As the TCR binds to its specific antigen presenting MHC molecule, sustained periods of engagement are necessary in order to adequately activate T cells to proliferate and carry out more complex functions. Thus, additional adhesion molecules are required in order to sustain this interface. It was shown that a 10, 000 fold increases in TCR antigen presentation required to induce proliferation in the presence of LFA-1/ICAM-1 interaction was still insufficient to induce proliferation in the absence of this

interaction (362,363). LFA-1 through a similar mechanism has also been shown to lower the threshold of B cell activation by facilitating B cell adhesion and synapse formation (361). Support for the co-stimulatory functions of LFA-1 also comes from studies demonstrating that LFA-1/ICAM-1 binding can lead to increased inositol phospholipid hydrolysis, appearance of the hyperphosphorylated p23 form of the TCR ζ chain, and sustained intracellular calcium levels and an increase in cytoplasmic calcium levels (361). Two distinct types of signaling have been recognized upon engagement of LFA-1 on T cells with its corresponding ligands on APC. First, is an inside-out signal which increases the avidity of the integrin. This signaling involves cytoplasmic proteins such as talin and cytohesin 1(364). The second signal is an outside-in signal believed to affect intracellular signalling (364). This process is believed to involve the transcription factor Jun activation domain binding protein or JAB-1 (shown to be involved in the cell cycle and regulation of the cytoskeleton), as well as cytohesin-1 (359,364,365). It has also recently been shown that engagement of LFA-1 with ICAM-1 in conjunction with TCR signaling greatly enhances Ras activation, an important regulator of T cell development, homeostasis, and proliferation. Overall, the net effect of these processes is a decrease in the threshold of T cell activation (300,366).

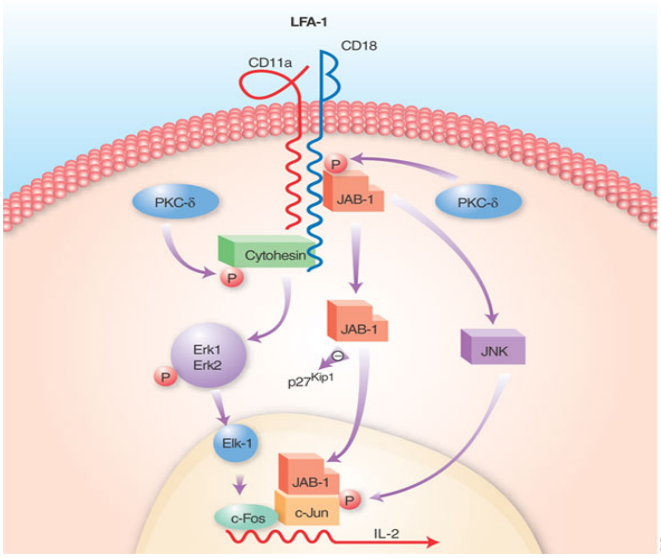

Figure 1.2: LFA-1 signaling pathways: LFA-1 engagement leads to two independent signaling pathways, both of which are dependent on PKCδ activity (360) .

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 (227,300,345,367-371). 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 (345,368). 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 mAb combined with anti-CD154 mAb has been shown to protect NPI xenografts (227), 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 (370,371). Anti-LFA-1 mAb has also been shown to be effective in islet xenograft models, both concordant and discordant. In a rat to mouse islet transplant model, Our group showed that short-term administration of anti-LFA-1 mAb prevented islet xenograft rejection for >100 days in 27 out of 28 recipients (372). In contrast, in a discordant xenograft model where NPI were transplanted into diabetic C57BL/6 mice, only 7/15 mice achieved normoglycemia, with only 6 of the 15 mice achieving long-term graft survival (227). This protection again however, was greatly enhanced with the addition of anti-CD154mAb to the anti-rejection regimen as 12/14 mice receiving NPI achieved long-term graft survival with this combination of mAbs (227). Recently a humanized IgG1 form of the anti-LFA-1 antibody (Efalizumab) which targets the CD11a alpha chain, has become available (373). Efalizumab has shown marked efficacy in the treatment of psoriasis and is currently in phase IV of clinical trials however, has failed to provide consistent effectiveness. Efalizumab treatment is associated with some side effects, which can include: headache, chills, fever, nausea, vomiting and myalgia, even more, higher doses were shown to increase the risk of developing lymphoproliferative disease. Following clinical reports, the FDA and Genentech Inc revised the safety warnings for this drug to include immune-mediated hemolytic anemia (373). Thus, though anti-LFA-1 mAb therapy has the potential to be a useful therapy, methods to improve its effectiveness with low doses, will need to be found (361). Currently, efalizumab is being tested as an anti-rejection therapy for islet transplant recipients.

1.5.3. Tolerance Induction

The goal of transplant immunology is to achieve a tolerance specific to the transplanted graft without administrating of general immunosuppressive drugs. 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 (374). There are two main approaches for the generation of tolerance, the generation of central tolerance and, the generation of peripheral tolerance. 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 (375). Immunological tolerance will be discussed further in section 1.6.

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 (376,377). 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 (378). 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 (378,379). 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 (380). While macroencapsulation devices are capable of restoring euglycemia in diabetic experimental animals (381-386), there are several shortcomings including: overgrowth resulting from poor biocompatibility, membrane rupture, insufficient release of insulin long term, and necrosis due to poor diffusion of nutrients to the islets (379,380). 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 (387-389). 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* (390). *In vivo*, several

groups have shown that encapsulation can successfully protect allo- and xenogeneic islets from immune mediated and autoimmune mediated rejection (224,379,391-394). 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 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 (224). 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 (387,389,395-397).

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 (398,399). 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, clusterin, and serine protease inhibitors (serpin)

(400-402), which are suspected to have immunoprotective, anti-inflammatory, and tolerizing properties (398,400-406).

1.6. IMMUNOLOGICAL TOLERANCE

Immune tolerance is a state in which the immune system is specifically unresponsive to antigens of interest. In the case of organ and cell transplantation, tolerance denotes a state of specific immune unresponsiveness to the donor graft, with normal responses to other antigens (407). The ability to respond normally to other antigens contrasts sharply with the effect of nonspecific immunosuppressive agents that are used clinically to prevent rejection, which are associated with increased risks of infection and malignancy. Extensive investigation of tolerance induction has occurred over more than 50 years since the first report of tolerance induction in mice by Billingham, Brent and Medawar (408). Although experimental tolerance induction has been tantalizing in both small and large animal transplant studies, significant challenges remain for clinical translation.

Achievement of transplantation tolerance is the “holy grail” in clinical transplantation for three major reasons. First, whilst improvements in nonspecific immunosuppressive therapy have markedly improved outcomes in organ transplantation, these drugs are associated with many specific organ toxicities as well as the life-long increased risks of infection and malignancy. Secondly, chronic rejection is a major factor contributing to constantly down sloping long-term survival curves for organ grafts. The half-lives of this second, late phase of graft loss have not changed significantly with improvements in

immunosuppressive therapy over the last 25 years. Chronic rejection can be avoided by tolerance induction. Thirdly, there is a critical shortage of allogeneic organs for transplantation, which could be overcome by the use of other species as organ and tissue sources, i.e. xenografts. However, immune barriers to xenografts look like even stronger than those to allografts, and the induction of tolerance at both the humoral and the cellular level is likely to be needed for the successful application of xenotransplantation in humans. The discovery of immunological tolerance may be considered a relatively recent event in history and could be attributed mainly to the independent experimental studies of Owen (409), Medawar and colleagues (408), and Hasek (410) along with the postulation of a natural state of immunological tolerance by Burnet and Fenner (411-413). Tolerance has been readily achievable by using two alternative approaches: Central tolerance and Peripheral tolerance.

1.6.1. Central Tolerance

T cell self-tolerance is attributed to a selection of T cells during or following their development, which involves the physical or functional elimination of those cells specific for self-antigens. Historically, central tolerance (tolerance induced in the thymus) has been considered to be largely responsible for shaping a T cell repertoire known for its specificity and its discriminatory nature. Thymocytes undergoing maturation and development in the thymus are subject to positive and negative selection. An overwhelming majority of thymocytes do not survive the selection events (414). In particular, negative

selection is important in regulating immune reactivity, as developing T cells bearing high-avidity TCRs specific for host antigens are eliminated in the thymus to prevent autoreactivity in the periphery. While studies relating to the autoimmune regulator (*Aire*) gene demonstrating the expression of peripheral tissue-specific antigens in the thymus provide renewed support for central tolerance as the sole necessary tolerance mechanism (415-418), they do not exclude the contribution by potential mechanisms of peripheral tolerance. The pattern of Aire-driven expression of peripheral tissue antigens by individual thymic medullary epithelial cells is highly heterogeneous, and is restricted both in terms of the frequency of cells that are able to present peripheral antigens and the number of antigens that individual cells express (419). Interestingly, thymic expression of certain peripheral tissue antigens is not driven by Aire (420). To make peripheral tolerance mechanisms dispensable, however, Aire and potentially other transcriptional regulators must clearly be able to regulate the expression of all relevant peripheral tissue antigens for which T cells specific for those antigens are able to recognize during development, but this remains unknown. Hence, tolerization of self-reactive T cells may occur during thymocyte maturation in the central lymphoid organs, as well as later in the periphery with mature T cells. What is not clear is whether these mechanisms, in addition to dealing with high-avidity T cells specific to peripheral antigens not present in the thymus, could also be responsible for governing low-avidity T cells that escape central tolerance (despite the presence of their cognate antigens in the thymus) (421,422).

1.6.2. Peripheral Tolerance

As indicated, some of the evidence in support of peripheral tolerance comes from transplant studies demonstrating the acceptance of an allogeneic graft given to the recipient as a peripheral tissue. However, the rules that govern the immunity/tolerance decision to a peripheral tissue-specific antigen have not yet been elucidated. An understanding of peripheral self-tolerance could lead to new avenues for therapeutic interventions in clinical transplantation of allogeneic cells/tissues for disease treatment. In particular, since $CD4^+$ T cells control a considerable portion of both B cell and $CD8^+$ T cell reactivity, elucidation of tolerance mechanisms in the $CD4^+$ compartment will be crucial to understanding tolerance in general (423-426). Peripheral tolerance in $CD4^+$ T cells represents a unique problem since these cells are only capable of recognizing antigen presented on the relatively few cell types that express MHC class II (APC) under normal circumstances, and the peptide antigens they recognize are derived largely from proteins taken up from the endocytic pathway, rather than from proteins made within the cell (427,428). Various models have been generated to explain the general rules determining peripheral $CD4^+$ T cell tolerance versus immunity and the specific mechanisms of tolerance involved.

1.6.3. Tolerance Induction in Transplantation

As mentioned earlier there are two main approaches for the induction of tolerance, the induction of central tolerance and, the induction of peripheral tolerance. Typically methods in transplantation aimed at induction of central

tolerance are chimerism induction and/or thymic grafting of donor antigens. In contrast, approaches that have focused on blocking costimulation, including the depletion of donor APC or host T cells, targeting of costimulatory/coinhibitory receptors/ligands by antibodies/fusion proteins, and removing the signals that lead to activation of APC and upregulation of costimulatory molecules, would be more relevant for understanding peripheral tolerance (375,407).

1.6.3.1. Mixed Chimerism

The most successful method of inducing transplantation tolerance is through the generation of systemic chimerism with donor cells. 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 (429,430). 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 (431). However, the tolerance in this situation is predominantly central rather than peripheral. 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 (189). Mixed chimerism in allo-transplant models has been firmly demonstrated, however inducing chimerism in a xenotransplant model has proven to be more challenging (432). 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 (433). The induction of mixed xenogeneic chimerism thereby prevents hyperacute rejection, a delayed antibody-mediated form of rejection termed acute vascular rejection, as well as cell-mediated rejection of primarily vascularized cardiac xenografts. Anti-Gal-producing cells are tolerized by an early anergy mechanism and later by clonal deletion and/or receptor editing. 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 attack and morbidity (434-436). Clearly, further investigation is needed with safety assurances and demonstrated effectiveness in larger animal models and before this therapy can advance to the clinic.

1.6.3.2. 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 (437,438). 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 (439). Intrathymic injection of allopeptides/allo-islets induces acquired tolerance in experimental animal models (440,441) and in some cases was successful in inducing permanent islet allograft survival (442,443). 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 (444). 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 (445). 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 (446). 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 (447).

1.6.3.3. Costimulation-Based Models

The discovery that T cell receptor stimulation without costimulation can induce tolerance has led to intensive evaluation of co-stimulatory blockade in the

transplantation field (310). Blockade of the CD28 co-stimulatory pathway can be achieved with specific mAb or with a soluble receptor for the B7-1／B7-2 ligands. Another pathway that has been targeted recently involves the interaction between CD154 on activated T cells with the CD40 receptor on APC. This interaction plays an important role in allowing APC to achieve full activating capacity by upregulating B7 molecules, MHC, antigen processing pathways, cytokines and other molecules. Blockade of the CD40–CD154 pathway alone or in combination with CTLA4-Ig can achieve marked prolongation of fully MHC-mismatched skin graft survival in some mouse strain combinations (448-450). However, permanent tolerance of these grafts is not reliably achieved. These treatments can more reliably induce permanent acceptance of tolerogenic rodent allografts such as hearts (451). Anergy of donor-reactive cells and an important role for Treg have been implicated in such models (452). Despite the achievement of prolonged allograft survival (though not tolerance) in nonhuman primates (332,453-455), attempts to apply co-stimulatory blockade for the induction of tolerance clinically have not succeeded.

1.6.3.4. Coinhibition -Based Models

The proposal that tolerance results from antigen encounter without an activating signal means antigen receptor engagement alone is a negative signal (tolerance is passively determined). Sinclair developed an alternative model in which the antigen receptor signal is positive. In this model costimulation serves to amplify an immune response rather than reverse an antigen receptor negative

signal; costimulation is counter balanced by receptors that mediate negative "coinhibitory" signals (tolerance is actively determined) (456). Coinhibition is a negative signal mediated by an antigen-nonspecific receptor working in concert with an antigen-specific signal through the antigen receptor. Under quiescent conditions, where costimulatory signals are limited, coinhibition may predominate (for example, due to the higher affinity of B7 for CTLA-4 compared to CD28) (457). Coinhibition was expanded to be part of a general model of immune regulation in T cells and B cells involving the balance between costimulation and coinhibition by numerous receptors, some examples include CTLA-4, Fas, PD-1, CD5, CD22, CD72 and interferon-gamma receptor. Thus, coinhibitory signals can be delivered by end products such as antibodies and cytokines or by receptor/ligand interactions between cells (458-460). There are now many studies that seem consistent with a role for coinhibitory receptors in peripheral $CD4^+$ T cell tolerance, as demonstrated by blocking the coinhibitor or eliminating it by gene knockout (461-465). Moreover, coinhibition seems to have become the paradigm for $CD4^+$ T cell tolerance and tolerance in general (466), and the signal 1 alone models of peripheral tolerance are clearly antiquated. However, there needs to be developed a clear definition of what controls coinhibition if we are to fully understand how it contributes to the immunity/tolerance decision or other potential functions it may have (467).

1.6.4. Mechanisms of T-Cell Tolerance

There are four major mechanisms of T-cell tolerance, including clonal deletion, anergy, ignorance and suppression (commonly referred to as "regulation"). These mechanisms may act alone or together to achieve tolerance and will be discussed in more details and in the context of transplantation.

1.6.4.1. Clonal Deletion

Clonal deletion implies death of T cells with receptors recognizing donor antigens. Deletion is the major mechanism of self-tolerance induction during T-cell development in the thymus (468-470). TCR with lower affinity for such complexes are more likely to survive this process, and other mechanisms are required to ensure their tolerance when they enter the periphery, particularly under conditions of inflammation and antigen upregulation (471-473). Mature T cells in the peripheral lymphoid tissues can also be deleted under certain conditions. Exposure of mature T cells to antigen in the periphery can also result in T-cell clonal deletion (474). Self antigen cross-presentation by lymph node dendritic cells under non-inflammatory conditions leads to deletion of tissue antigen-specific $CD8^+$ cytotoxic T cells (CTL) (475). $CD8^+$ cells may be deleted because of "exhaustion" in the presence of a large, persistent antigen load (476). As an alternative to global T-cell depletion, costimulatory blockade with anti-CD154 can be used in combination with bone marrow transplantation (BMT) to achieve mixed chimerism and long-term central, deletional tolerance (477,478). In such animals, the preexisting alloreactive T-cell repertoire is not depleted with

mAb, and other mechanisms come into play. A similar phenomenon has been demonstrated for peripheral CD8 cells in mice receiving donor-specific transfusion (DST) combined with anti-CD154 (479). Peripheral T cell apoptosis has been demonstrated, though without specific markers for alloreactive T cells, in mice tolerized with anti-CD154 mAb, rapamycin and cardiac allografts (480). Recently, $CD4^{-}CD8^{-}$ cytotoxic regulatory cells have been reported to delete alloreactive $CD8^{+}$ T cells with the same specificity as the regulatory cells (481).

1.6.4.2. Anergy

Anergy denotes the inability of T cells to proliferate and produce interleukin-2 (IL-2) in response to antigens they recognize. T cell anergy develops when T cells encounter peptide/MHC complexes without receiving adequate accessory or costimulatory signals (482). T cells can also be rendered anergic if they encounter peptide ligands for which they have low affinity (469). Certain APC, such as macrophages and tolerogenic dendritic cells that may be immature or matured in a specific manner have the capacity to induce T cell anergy, in part due to secretion of suppressive cytokines and lack of adequate costimulation (483,484). Anergy is associated with altered signalling and tyrosine phosphorylation patterns (482,485). T cell anergy can often (486), but not always (487,488), be overcome by providing exogenous IL-2. Anergy has been associated with TCR down-modulation (489). It should be borne in mind that anergy is reversible under pro-inflammatory conditions, including the presence of infection, so it is unlikely to be reliable as the sole long-term tolerance mechanism

(490,491). Deletion has followed induction of an anergic state in the continued presence of antigen in some, but not all, models (492,493). Anergic T cells may also down-regulate the activity of other T cells, so that they function as regulatory T cells (Treg), perhaps by conditioning APC such that they tolerize T cells recognizing the same or different antigens presented by these APC (494). Moreover, regulatory T cells can promote the induction of T cell anergy and may themselves have biochemical properties suggestive of an anergic state (495,496).

1.6.4.3. Ignoring Graft Antigens (Ignorance)

In some situations, antigens may simply be ignored by T cells (489) or B cells (497) with receptors recognizing them. This may occur when antigens are presented by "nonprofessional APC" which are unable to activate T cells, or when T cells fail to migrate to the antigen-bearing tissue, as documented in murine solid tumour models (498). Several factors appear to determine such T cell behaviour, including the level of antigen expression, how recently the responding T cell has emerged from the thymus (489), and the presence or absence of proinflammatory cytokines (499) and co-stimulatory molecules in peripheral tissues (500). As might be easily imagined, ignorance is a precarious state which can be upset by additional immunological stimuli provoked by inflammation induced by infections or by presentation of antigen on professional APC (407,501,502).

1.6.4.4. Active Suppression of T-Cell Responses (Regulation)

Suppression, in which a cell population actively down-regulates the reactivity of T cells, has recently been implicated in many rodent transplantation tolerance models and in the maintenance of self-tolerance. It has become increasingly clear in recent years that several mechanisms exist to down-modulate immune responses once they are initiated, and that it is the balance of activating and modulating functions that determine the outcome of any response. Many mechanisms, including killing of APC by CTL, inhibitory effects of cytokines, activation-induced cell death, contribute to this down-modulation of immune responses. In addition, studies in the 1970s introduced the concept that T cells themselves could actively suppress immune responses. Whilst certain T cell and non-T cell populations were implicated in this suppression, it is only in the last decade or so that molecular markers of suppressive T cells have been identified and that suppressive cell populations have been isolated, cultured *in vitro* and adoptively transferred (407,468,471,503,504).

1.6.4.4.1. Regulatory $CD4^+$ T cells

There has been an huge expansion of information and studies involving immunoregulatory cells in recent years. Numerous types of cells with a regulatory phenotype have been identified. 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 (505-509). Suppressive

$CD4^+CD25^+$ T cells have been strongly implicated in the induction and maintenance of self-tolerance (510-514). Functional evidence for specific suppressor cells was obtained in early models of transplantation tolerance (515) and Hall *et al.* first identified $CD4^+CD25^+$ T cells as a specific suppressive population in rats receiving cardiac allografts with a short course of cyclosporine (516). Since then, Treg have been implicated in numerous models involving acceptance of vascularized allografts in rodents receiving an initial immunosuppressive treatment (517-522). 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 (514,523,524). 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* (525). More recent studies have shown that these cells are generated mainly in the thymus, require specific positive selection (471) and express Forkhead box P3 (FoxP3), a transcription factor that controls the genetic program associated with their suppressive activity (526,527). *In vitro* suppression by these T regulatory cells (T reg) seems to require cell-to-cell contact (528). Transforming growth factor-β (TGF-β) is a cytokine that has been strongly implicated in the maintenance of Treg and as a mediator of their suppressive activity (529-531). Both $CD4^+$ and $CD8^+$ T cells are subject to suppression by Treg, and memory as well as naïve responses have been shown to be suppressed. Several reports indicate that Treg require specific antigen for their activation, but that the final effector mechanism of suppression is nonantigen

specific (517,532,533). Rechallenge with specific antigen induces γ-interferon (IFN-γ) expression by Treg, which appears to be critical for their function (534). Generation, expansion, survival and possibly the function of Treg is highly dependent on IL-2, which is not produced by the Treg themselves (535). Additional $CD4^+$ T cell populations with suppressive function include $FoxP3^+$ $CD25^+$ cells that arise from $FoxP3^-CD25^-$ cells in the periphery following antigen-specific stimulation ("adaptive" Treg) (528), especially in the presence of TGF-β (536). Additionally, one type of T reg (type 1;'Tr1) are induced by chronic antigenic stimulation in the presence of IL-10 and can suppress autoimmune diseases in mice. These cells produce high levels of IL-10 and low amounts of IL-2 (537), and immature dendritic cells can support their development *in vitro* (538). Both natural Treg and Tr1 cells are hyporesponsive to TCR-mediated stimulation but can be grown slowly *in vitro* in the presence of certain cytokines, including IL-2. The *in vitro* suppressive function of Tr1 is dependent on IL-10 and TGF-β (537). TGF-β is clearly an important cytokine for several suppressive populations. Besides maintaining peripheral Treg populations and functions (531,539), TGF-β promotes adaptive Treg differentiation (540) and suppresses T-cell activation and T_H1 differentiation through several Treg-independent mechanisms (539,541). It can also modulate dendritic cell function, rendering them tolerogenic for T cells (483). Suppressive T cells have been implicated in numerous experimental models leading to allograft tolerance (542,543). There is considerable evidence for a role for natural Treg in maintaining self-tolerance in humans. Congenital defects in FoxP3 in humans are associated with an autoimmune syndrome,

immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX), that resembles its counterpart in mice (the 'scurfy' mutant) (544). Defects in IL-2 signalling through the STAT-5 transcription factor lead to similar defects in Treg in mice and humans (545,546). These and the above experimental results have led to considerable interest in the role of Treg in clinical transplantation, and correlative data have begun to emerge.

1.6.4.4.2. Other suppressive cell populations

In addition to the $CD4^+CD25^+$ T cell populations discussed above, other T-cell and non-T-cell suppressive cell populations can down-modulate immune responses. Fully differentiated $CD4^+$ helper (T_H) cells may polarize their cytokine secretion patterns to that of the T_H1 subset, which secretes IL-2 and IFN-γ, the Th17 subset that produces IL-17 (407,537) or the T-helper type 2 (T_H2) subset that secretes IL-4 and IL-10 (547). T_H1 cells promote the generation of cytolytic $CD8^+$ T cells, whilst T_H2 helps antibody responses but not CTL responses (547). A similar polarization of the pattern of cytokine secretion occurs in $CD8^+$ cytolytic T cells (548). In the early 1990s, there was considerable interest in the concept that polarization to T_H2 type of response from a pro-inflammatory T_H1 (IL-2- and IFN-γ-producing) response could promote allograft acceptance, and data associated T_H2 responses with such acceptance (549). However, only a few studies directly demonstrated a role for T_H2 cells in tolerance induction and it is now clear that T_H2 cells and their cytokines can promote allograft rejection (549). Natural killer (NK) T cells (T cells that express NK cell-associated markers and

may utilize an invariant TCR-α chain) are another subset of T cells with regulatory activity, which may be mediated in part by T_H2-type cytokines (550). NKT cells have recently been shown to depend on TGF-β for their development. NKT cells are enriched in bone marrow and can suppress GVHD (551,552), at least in part via an IL-4-dependent mechanism (552). A $CD4^-CD8^-$ T cell population lacking NK cell markers that suppresses skin graft rejection by CD8 T cells with the same TCR has been described in a mouse model (481), but the importance of this cell population in other settings remains to be determined. Human $CD8^+CD28^-$ T cells have been reported to suppress alloresponses and xenoresponses *in vitro* (553), and recent studies have implicated $CD8^+$ T cells as regulatory cells in models of autoimmunity (554), heart graft acceptance(555), skin grafting (556) and GVHD (557-559). 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 (560). "Natural"(559) and "adaptive"(557), FoxP3-expressing (555,559), TGF-β-producing(561), and IL-10-producing (557) regulatory $CD8^+$ T cells have been described, and extensive data are emerging on the role of these cells in various models. One mechanism of immune down-modulation mediated by $CD8^+$ T cells is simply the killing by alloreactive CTL of critical donor APC populations (562). Some $CD8^+$ CTL-mediated suppressive phenomena might be attributable to "veto" activity of these cells(563). Veto cells inactivate CTL recognizing antigens expressed on the veto cell surface(563), resulting in suppression of CTL responses to antigens shared by the veto cells. CTL, various bone marrow cell subsets and

NK cells have been reported to have such activity (563). Veto cells may promote GVH tolerance, promote allogeneic marrow engraftment and promote tolerance induction with DST (515,563). Veto activity has been suggested to involve TGF-β (564). Thus, whilst many types of Treg have been recently described, much remains to be learned about the relative importance of each of these, their potential in large animal models and the circumstances under which they can be optimally generated. Several groups are exploring the approach of expanding Treg *in vitro* and then administering them *in vivo* to suppress alloimmunity or autoimmunity. Whilst methods of nonspecifically (565,566) expanding mouse and human Treg *ex vivo* have recently been developed, animal studies suggest that antigen specificity is important for the achievement of effective suppression following adoptive transfer (566). As alloreactivity includes many different donor antigens and donor cells will not be available pretransplant for cadaveric donor transplantation, this approach may be difficult to apply (407,566).

1.7. OBJECTIVES AND GENERAL OUTLINE

Islet transplantation is a more physiological treatment alternative to T1DM. Unfortunately at present, the number of patients who could benefit from this therapy hugely 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 particularly children with type 1 diabetes. Xenotransplantation,

using NPI as a source of tissue for transplantation has the potential to improve the supply shortage of donor islets. It was shown by our group that transient perturbation of adhesion and co-stimulatory pathways using short-term administrations of a combination of anti-LFA-1 and anti-CD154 mAbs is highly effective in preventing NPI xenografts from rejection in B6 mice (227). We hypothesized that this combined mAb therapy could induce tolerance to NPI xenografts. Therefore the main objective of this thesis is to examine whether a combination of anti-LFA-1 and anti-CD154 mAbs could induce tolerance to NPI xenografts. If so, the mechanism of induction and maintenance of tolerance will also be investigated.

In chapter 1 an introduction to diabetes mellitus, the field of islet transplantation, porcine islet xenotransplnatation, strategies for preventing immune rejection of tarnsplnated islets and immunological tolerance was presented. In Chapter 2, we determined whether this combination of mAb therapy could induce tolerance to NPI xenografts in B6 mice. In Chapter 3, we determined the specificity (species and tissue) of tolerance to NPI xenografts. We also investigated if tolerance could be extended to second party porcine islet xenografts in case human islet transplant recipients would require a second islet transplant to maintain insulin independence. We also determined if tolerance could be extended to different tissue or organ grafts (i.e. skin grafts) without any additional anti-rejection therapy. It is important because patients with type 1 diabetes are susceptible to developing deleterious secondary tissue or organ complications that may require replacement by transplantation.

In chapter 4, we determined if tolerance induced is dominant and also determined the role of $CD4^+$ and $CD8^+$ T cell substes in the induction and maintenance of tolerance to NPI xenografts. In chapter 5, we determined that PD-1/PD-1L pathway is required for the induction and maintenance of tolerance to NPI xenografts by combined anti-LFA-1 and anti-CD154 mAbs. Lastly, in chapter 6 we determined the efficacy of combined anti-LFA-1 and anti-CD154 mAb therapy in preventing the rejection of NPI xenografts in autoimmune prone NOD mice, which is an animal model of type 1 diabetes.

We hope that with the development of safe and effective anti-rejection agents which target adhesion and costimulatory pathways of T cell activation, the goal of widespread application of islet transplantation particularly for children with type 1 diabetes could be achieved.

1.8. REFERENCES

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

2. Kasper D AFDLESHaJJ, ed.Harrison's Principles of InternalMedicine - 16th Ed.USA: McGraw-Hill Companies: Harrison's Principles of Internal Medicine. New York, Knopf: Distributed by Random House, 2005,

3. The Canon of Medicine" (work by Avicenna). http://www.britannica.com/eb/topic-92902/The-Canon-of-Medicine . 2008.

4. Sakula,A: Paul Langerhans (1847-1888): a centenary tribute. *J.R.Soc.Med.* 81:414-415, 1988

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

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

7. The prevalence and costs of diabetes (Canadian Diabetes Association, 2005-2009). http://www.diabetes.ca/about-diabetes/what/prevalence/ . 2009.

8. Total Prevalence of Diabetes & Pre-diabetes (American Diabetes Association). http://www.diabetes.org/diabetes-statistics/prevalence.jsp . 2008.

9. Harris,MI, Flegal,KM, Cowie,CC, Eberhardt,MS, Goldstein,DE, Little,RR, Wiedmeyer,HM, Byrd-Holt,DD: Prevalence of diabetes, impaired fasting glucose, and impaired glucose tolerance in U.S. adults. The Third National Health and Nutrition Examination Survey, 1988-1994. *Diabetes Care* 21:518-524, 1998

10. Kraut,A, Walld,R, Tate,R, Mustard,C: Impact of diabetes on employment and income in Manitoba, Canada. *Diabetes Care* 24:64-68, 2001

11. Rubin,RR, Peyrot,M: Quality of life and diabetes. *Diabetes Metab Res.Rev.* 15:205-218, 1999

12. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 27 Suppl 1:S5-S10, 2004

13. Diabetes in Canada, Chapter 5 Use of Health Services and Costs, Public Health Agency of Canada. http://www.phac-aspc.gc.ca/publicat/dic-dac2/english/36chap5-eng.php . 2009.

14. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 26 Suppl 1:S5-20, 2003

15. Pinhas-Hamiel,O, Zeitler,P: Clinical presentation and treatment of type 2 diabetes in children. *Pediatr.Diabetes* 8 Suppl 9:16-27, 2007

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

17. Haller,MJ, Atkinson,MA, Schatz,D: Type 1 diabetes mellitus: etiology, presentation, and management. *Pediatr.Clin.North Am.* 52:1553-1578, 2005

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

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

20. Schipper,RF, Koeleman,BP, Bruining,GJ, Schreuder,GM, Verduijn,W, De Vries,RR, Roep,BO: HLA class II associations with Type 1 diabetes mellitus: a multivariate approach. *Tissue Antigens* 57:144-150, 2001

21. Roep,BO, Schipper,R, Verduyn,W, Bruining,GJ, Schreuder,GM, De Vries,RR: 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, 1999

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

23. Van der,AB, Van Waeyenberge,C, Schuit,F, Heimberg,H, Vandewalle,C, Gorus,F, Flament,J: 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, 1995

24. Kim,MS, Polychronakos,C: Immunogenetics of type 1 diabetes. *Horm.Res.* 64:180-188, 2005

25. Maier,LM, Wicker,LS: Genetic susceptibility to type 1 diabetes. *Curr.Opin.Immunol.* 17:601-608, 2005

26. Nepom,GT, Kwok,WW: Molecular basis for HLA-DQ associations with IDDM. *Diabetes* 47:1177-1184, 1998

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

28. Jahromi,MM, Eisenbarth,GS: Cellular and molecular pathogenesis of type 1A diabetes. *Cell Mol.Life Sci.* 64:865-872, 2007

29. Peng,H, Hagopian,W: Environmental factors in the development of Type 1 diabetes. *Rev.Endocr.Metab Disord.* 7:149-162, 2006

30. Karounos,DG, Wolinsky,JS, Thomas,JW: Monoclonal antibody to rubella virus capsid protein recognizes a beta-cell antigen. *J.Immunol.* 150:3080-3085, 1993

31. Kaufman,DL, Erlander,MG, Clare-Salzler,M, Atkinson,MA, Maclaren,NK, Tobin,AJ: Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus. *J.Clin.Invest* 89:283-292, 1992

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

33. Hiemstra,HS, Schloot,NC, van Veelen,PA, Willemen,SJ, Franken,KL, van Rood,JJ, De Vries,RR, Chaudhuri,A, Behan,PO, Drijfhout,JW, Roep,BO: Cytomegalovirus in autoimmunity: T cell crossreactivity to viral antigen and autoantigen glutamic acid decarboxylase. *Proc.Natl.Acad.Sci.U.S.A* 98:3988-3991, 2001

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

35. Scott,FW, Norris,JM, Kolb,H: Milk and type I diabetes. *Diabetes Care* 19:379-383, 1996

36. Norris,JM, Scott,FW: A meta-analysis of infant diet and insulin-dependent diabetes mellitus: do biases play a role? *Epidemiology* 7:87-92, 1996

37. Borch-Johnsen,K, Joner,G, Mandrup-Poulsen,T, Christy,M, Zachau-Christiansen,B, Kastrup,K, Nerup,J: Relation between breast-feeding and

incidence rates of insulin-dependent diabetes mellitus. A hypothesis. *Lancet* 2:1083-1086, 1984

38. Paronen,J, Knip,M, Savilahti,E, Virtanen,SM, Ilonen,J, Akerblom,HK, Vaarala,O: 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, 2000

39. Vaarala,O, Knip,M, Paronen,J, Hamalainen,AM, Muona,P, Vaatainen,M, Ilonen,J, Simell,O, Akerblom,HK: Cow's milk formula feeding induces primary immunization to insulin in infants at genetic risk for type 1 diabetes. *Diabetes* 48:1389-1394, 1999

40. Vaarala,O, Paronen,J, Otonkoski,T, Akerblom,HK: 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, 1998

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

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

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

44. Kostraba,JN, Gay,EC, Rewers,M, Hamman,RF: Nitrate levels in community drinking waters and risk of IDDM. An ecological analysis. *Diabetes Care* 15:1505-1508, 1992

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

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

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

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

49. Scott,FW, Sarwar,G, Cloutier,HE: Diabetogenicity of various protein sources in the diet of the diabetes-prone BB rat. *Adv.Exp.Med.Biol.* 246:277-285, 1988

50. Yoon,JW, Jun,HS: Cellular and molecular pathogenic mechanisms of insulin-dependent diabetes mellitus. *Ann.N.Y.Acad.Sci.* 928:200-211, 2001

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

52. Francine R.Kaufman: Medical Management of Type 1 Diabetes. American Diabetes Association., 2008,

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

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

55. Ricordi C: Pancreatic Islet Cell Transplantation, Austin: R.G Landes Company. 1992,

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

57. DOCHERTY KADS. The Molecular and Cell Biology of the Beta Cell. In: Porte Jr D, R Sherwin & A Baron, ed. Ellenberg & Rifkin's Diabetes Mellitus. New York: McGraw-Hill. 2003.

58. KAHN CRS, ALAN R. The Molecular Mechanism of Insulin Action and the Regulation of Glucose and Lipid Metabolism. In: Kahn CRK, George L.; Moses, Alan C.; Weir, Gordon C.; Jacobson, Alan M.; Smith, Robert J, ed. Joslin's Diabetes Mellitus. Boston: Lippincott Williams & Wilkins. 2005.

59. Suckale,J, Solimena,M: Pancreas islets in metabolic signaling--focus on the beta-cell. *Front Biosci.* 13:7156-7171, 2008

60. Schade,DS, Eaton,RP: Prevention of diabetic ketoacidosis. *JAMA* 242:2455-2458, 1979

61. Schade,DS, Eaton,RP: Pathogenesis of diabetic ketoacidosis: a reappraisal. *Diabetes Care* 2:296-306, 1979

62. Chernick,SS, Clark,CM, Jr., Gardiner,RJ, Scow,RO: Role of lipolytic and glucocorticoid hormones in the development of diabetic ketosis. *Diabetes* 21:946-954, 1972

63. Nair,KS, Garrow,JS, Ford,C, Mahler,RF, Halliday,D: Effect of poor diabetic control and obesity on whole body protein metabolism in man. *Diabetologia* 25:400-403, 1983

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

65. POWERS A. Diabetes Mellitus. In: Kasper D, AS Fauci, DL Longo, E Braunwald, SL Hauser and JL Jameson, ed. Harrison's Principles of Internal Medicine - 16th Ed. USA: McGraw-Hill Companies. 2005.

66. Jaremko,J, Rorstad,O: Advances toward the implantable artificial pancreas for treatment of diabetes. *Diabetes Care* 21:444-450, 1998

67. BLISS M. Banting A Biography. Toronto: University of Toronto Press. 1992.

68. BRAR D. The History of Insulin. Berkeley. 2006.

69. Gerich,JE: Novel insulins: expanding options in diabetes management. *Am.J.Med.* 113:308-316, 2002

70. Matthews, D. Diabetes, (Oxford University Press, Oxford ; New York). 2008.

71. Skyler,JS, Cefalu,WT, Kourides,IA, Landschulz,WH, Balagtas,CC, Cheng,SL, Gelfand,RA: Efficacy of inhaled human insulin in type 1 diabetes mellitus: a randomised proof-of-concept study. *Lancet* 357:331-335, 2001

72. DeWitt,DE, Hirsch,IB: Outpatient insulin therapy in type 1 and type 2 diabetes mellitus: scientific review. *JAMA* 289:2254-2264, 2003

73. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. *N.Engl.J.Med.* 329:977-986, 1993

74. Reichard,P, Nilsson,BY, Rosenqvist,U: The effect of long-term intensified insulin treatment on the development of microvascular complications of diabetes mellitus. *N.Engl.J.Med.* 329:304-309, 1993

75. RICORDI C.: Pancreatic Islet Cell Transplantation. Austin, R.G Landes Company, 1992,

76. SUTHERLAND D, RWG GRUESSNER, , SM MAUER, and AC GRUESSNER. Pancreas Transplantation. In: Porte Jr D, R Sherwin & A Baron, ed. Ellenberg & Rifkin's Diabetes Mellitus. New York: McGraw-Hill. 2003.

77. GROTH CG. Pancreatic Transplantation. Philadelphia: W.B. Saunders Company. 1988.

78. Kelly,WD, Lillehei,RC, Merkel,FK, Idezuki,Y, Goetz,FC: Allotransplantation of the pancreas and duodenum along with the kidney in diabetic nephropathy. *Surgery* 61:827-837, 1967

79. Cohen,DJ, St Martin,L, Christensen,LL, Bloom,RD, Sung,RS: Kidney and pancreas transplantation in the United States, 1995-2004. *Am.J.Transplant.* 6:1153-1169, 2006

80. Sutherland,DE, Gruessner,RW, Dunn,DL, Matas,AJ, Humar,A, Kandaswamy,R, Mauer,SM, Kennedy,WR, Goetz,FC, Robertson,RP, Gruessner,AC, Najarian,JS: Lessons learned from more than 1,000 pancreas transplants at a single institution. *Ann.Surg.* 233:463-501, 2001

81. Gruessner,RW, Sutherland,DE, Najarian,JS, Dunn,DL, Gruessner,AC: Solitary pancreas transplantation for nonuremic patients with labile insulin-dependent diabetes mellitus. *Transplantation* 64:1572-1577, 1997

82. Bartlett,ST, Schweitzer,EJ, Johnson,LB, Kuo,PC, Papadimitriou,JC, Drachenberg,CB, Klassen,DK, Hoehn-Saric,EW, Weir,MR, Imbembo,AL: Equivalent success of simultaneous pancreas kidney and solitary pancreas transplantation. A prospective trial of tacrolimus immunosuppression with percutaneous biopsy. *Ann.Surg.* 224:440-449, 1996

83. Ryan,EA, Bigam,D, Shapiro,AM: Current indications for pancreas or islet transplant. *Diabetes Obes.Metab* 8:1-7, 2006

84. Sutherland,DE: Pancreas transplants. *Br.J.Surg.* 81:2-4, 1994

85. Humar,A, Kandaswamy,R, Granger,D, Gruessner,RW, Gruessner,AC, Sutherland,DE: Decreased surgical risks of pancreas transplantation in the modern era. *Ann.Surg.* 231:269-275, 2000

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

87. Venstrom,JM, McBride,MA, Rother,KI, Hirshberg,B, Orchard,TJ, Harlan,DM: Survival after pancreas transplantation in patients with diabetes and preserved kidney function. *JAMA* 290:2817-2823, 2003

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

89. Kroon,E, Martinson,LA, Kadoya,K, Bang,AG, Kelly,OG, Eliazer,S, Young,H, Richardson,M, Smart,NG, Cunningham,J, Agulnick,AD, D'Amour,KA, Carpenter,MK, Baetge,EE: Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. *Nat.Biotechnol.* 26:443-452, 2008

90. Shi,Y, Hou,L, Tang,F, Jiang,W, Wang,P, Ding,M, Deng,H: 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, 2005

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

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

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

94. Best,M, Carroll,M, Hanley,NA, Piper,HK: Embryonic stem cells to beta-cells by understanding pancreas development. *Mol.Cell Endocrinol.* 288:86-94, 2008

95. Liu,M, Han,ZC: Mesenchymal stem cells: biology and clinical potential in type 1 diabetes therapy. *J.Cell Mol.Med.* 12:1155-1168, 2008

96. Bonner-Weir,S, Taneja,M, Weir,GC, Tatarkiewicz,K, Song,KH, Sharma,A, O'Neil,JJ: In vitro cultivation of human islets from expanded ductal tissue. *Proc.Natl.Acad.Sci.U.S.A* 97:7999-8004, 2000

97. Ramiya,VK, Maraist,M, Arfors,KE, Schatz,DA, Peck,AB, Cornelius,JG: Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. *Nat.Med.* 6:278-282, 2000

98. Zulewski,H, Abraham,EJ, Gerlach,MJ, Daniel,PB, Moritz,W, Muller,B, Vallejo,M, Thomas,MK, Habener,JF: Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. *Diabetes* 50:521-533, 2001

99. Huang,H, Tang,X: Phenotypic determination and characterization of nestin-positive precursors derived from human fetal pancreas. *Lab Invest* 83:539-547, 2003

100. Vinik,AI, Fishwick,DT, Pittenger,G: Advances in diabetes for the millennium: toward a cure for diabetes. *MedGenMed.* 6:12, 2004

101. Yoon,JW, Jun,HS: Recent advances in insulin gene therapy for type 1 diabetes. *Trends Mol.Med.* 8:62-68, 2002

102. Heit,JJ, Kim,SK: Embryonic stem cells and islet replacement in diabetes mellitus. *Pediatr.Diabetes* 5 Suppl 2:5-15, 2004

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

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

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

106. Bernard-Kargar,C, Ktorza,A: Endocrine pancreas plasticity under physiological and pathological conditions. *Diabetes* 50 Suppl 1:S30-S35, 2001

107. Lipsett,M, Finegood,DT: beta-cell neogenesis during prolonged hyperglycemia in rats. *Diabetes* 51:1834-1841, 2002

108. Stocum,DL: Development. A tail of transdifferentiation. *Science* 298:1901-1903, 2002

109. Arnush,M, Gu,D, Baugh,C, Sawyer,SP, Mroczkowski,B, Krahl,T, Sarvetnick,N: Growth factors in the regenerating pancreas of gamma-interferon transgenic mice. *Lab Invest* 74:985-990, 1996

110. Bonner-Weir,S, Deery,D, Leahy,JL, Weir,GC: Compensatory growth of pancreatic beta-cells in adult rats after short-term glucose infusion. *Diabetes* 38:49-53, 1989

111. Nir,T, Melton,DA, Dor,Y: Recovery from diabetes in mice by beta cell regeneration. *J.Clin.Invest* 117:2553-2561, 2007

112. Sutherland,DE, Sibley,R, Xu,XZ, Michael,A, Srikanta,AM, Taub,F, Najarian,J, Goetz,FC: Twin-to-twin pancreas transplantation: reversal and reenactment of the pathogenesis of type I diabetes. *Trans.Assoc.Am.Physicians* 97:80-87, 1984

113. Anderson,MS, Bluestone,JA: The NOD mouse: a model of immune dysregulation. *Annu.Rev.Immunol.* 23:447-485, 2005

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

115. Suarez-Pinzon,WL, Lakey,JR, Brand,SJ, Rabinovitch,A: 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, 2005

116. Cerco Medical: Science: Methods. http://www.isletmedical.com/pages/define_methods.htm . 2009.

117. Alejandro,R, Lehmann,R, Ricordi,C, Kenyon,NS, Angelico,MC, Burke,G, Esquenazi,V, Nery,J, Betancourt,AE, Kong,SS, Miller,J, Mintz,DH: Long-term function (6 years) of islet allografts in type 1 diabetes. *Diabetes* 46:1983-1989, 1997

118. Shapiro, A. M. J and Shaw, J. A. M. Islet transplantation and beta cell replacement therapy, (Informa Healthcare, New York). 2007.

119. Von Mering J, ,MO: Diabetes mellitus after pancreas extirpation. *Archiv fur Experimentale Pathologie und Pharmacologie* 26:111-127, 1889

120. Minkowski O, .Weitere Mitteilungen ü ber den: Diabetes mellitus nach Extirpation des Pankreas. *Berl Klin Wochenschr* 29:90-93, 1892

121. Williams P.: Notes on diabetes treated with extract and by grafts of sheep's pancreas. *Br Med J.1* 2:1303-1304, 1894

122. HELLERSTROEM,C: A METHOD FOR THE MICRODISSECTION OF INTACT PANCREATIC ISLETS OF MAMMALS. *Acta Endocrinol.(Copenh)* 45:122-132, 1964

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

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

125. Lindall,A, Steffes,M, Sorenson,R: Immunoassayable insulin content of subcellular fractions of rat islets. *Endocrinology* 85:218-223, 1969

126. Scharp,DW, Kemp,CB, Knight,MJ, Ballinger,WF, Lacy,PE: The use of ficoll in the preparation of viable islets of langerhans from the rat pancreas. *Transplantation* 16:686-689, 1973

127. YOUNOSZAI R, SORENSON RL, LINDALL AW.: Homo-transplantation of isolated pancreatic islets. *Diabetes* 19:406, 1970

128. Ballinger,WF, Lacy,PE: Transplantation of intact pancreatic islets in rats. *Surgery* 72:175-186, 1972

129. Scharp,DW, Downing,R, Merrell,RC, Greider,M: Isolating the elusive islet. *Diabetes* 29 Suppl 1:19-30, 1980

130. Gray,DW, McShane,P, Grant,A, Morris,PJ: A method for isolation of islets of Langerhans from the human pancreas. *Diabetes* 33:1055-1061, 1984

131. Reckard,CR, Ziegler,MM, Barker,CF: Physiological and immunological consequences of transplanting isolated pancreatic islets. *Surgery* 74:91-99, 1973

132. Lorenz,D, Rosenbaum,KD, Petermann,J, Ziegler,M, Beckert,R, Dorn,A: Transplantation of isologous islets of Langerhans in diabetic rats. *Acta Diabetol.Lat.* 12:30-40, 1975

133. Lazarow,A, Wells,LJ, Carpenter,AM, Hegre,OD, Leonard,RJ, McEvoy,RC: The Banting Memorial Lecture 1973: Islet differentiation, organ culture, and transplantation. *Diabetes* 22:877-912, 1973

134. Reckard,CR, Barker,CF: Transplantation of isolated pancreatic islets across strong and weak histocompatibility barriers. *Transplant.Proc.* 5:761-763, 1973

135. Panijayanond,P, Soroff,HS, Monaco,AP: Pancreatic islet isografts in mice. *Surg.Forum* 24:329-331, 1973

136. Kemp,CB, Knight,MJ, Scharp,DW, Ballinger,WF, Lacy,PE: Effect of transplantation site on the results of pancreatic islet isografts in diabetic rats. *Diabetologia* 9:486-491, 1973

137. Henriksson,C, Bergmark,J, Claes,G: Metabolic response to isologous transplantation of small numbers of isolated islets of Langerhans in the rat. *Eur.Surg.Res.* 9:411-418, 1977

138. Rumpf,KD, Lohlein,D, Pichlmayr: Multiple transplantation of islets of Langerhans. *Eur.Surg.Res.* 9:403-410, 1977

139. Matas,AJ, Payne,WD, Grotting,JC, Sutherland,DE, Steffes,MW, Hertel,BF, Najarian,JS: Portal versus systemic transplantation of dispersed neonatal pancreas. *Transplantation* 24:333-337, 1977

140. Reckard,CR, Franklin,W, Schulak,JA: Intrasplenic versus intraportal pancreatic islet transplants: quantitative, qualitative and immunological aspects. *Trans.Am.Soc.Artif.Intern.Organs* 24:232-234, 1978

141. Finch,DR, Wise,PH, Morris,PJ: Successful intra-splenic transplantation of syngeneic and allogeneic isolated pancreatic islets. *Diabetologia* 13:195-199, 1977

142. Cuthbertson,RA, Mandel,TE: A comparison of portal versus systemic venous drainage in murine foetal pancreatic islet transplantation. *Aust.J.Exp.Biol.Med Sci.* 64 (Pt 2):175-184, 1986

143. Kin,T, Korbutt,GS, Kobayashi,T, Dufour,JM, Rajotte,RV: Reversal of diabetes in pancreatectomized pigs after transplantation of neonatal porcine islets. *Diabetes* 54:1032-1039, 2005

144. Ao,Z, Matayoshi,K, Yakimets,WJ, Katyal,D, Rajotte,RV, Warnock,GL: Development of an omental pouch site for islet transplantation. *Transplant.Proc.* 24:2789, 1992

145. Yasunami,Y, Lacy,PE, Finke,EH: A new site for islet transplantation--a peritoneal-omental pouch. *Transplantation* 36:181-182, 1983

146. Reece-Smith,H, McShane,P, Morris,PJ: Glucose and insulin changes following a renoportal shunt in streptozotocin diabetic rats with pancreatic islet isografts under the kidney capsule. *Diabetologia* 23:343-346, 1982

147. Reece-Smith,H, Du Toit,DF, McShane,P, Morris,PJ: Prolonged survival of pancreatic islet allografts transplanted beneath the renal capsule. *Transplantation* 31:305-306, 1981

148. Brown,J, Mullen,Y, Clark,WR, Molnar,IG, Heininger,D: Importance of hepatic portal circulation for insulin action in streptozotocin-diabetic rats transplanted with fetal pancreases. *J.Clin.Invest* 64:1688-1694, 1979

149. Gray,DW, Reece-Smith,H, Fairbrother,B, McShane,P, Morris,PJ: Isolated pancreatic islet allografts in rats rendered immunologically unresponsive

to renal allografts. The effect of the site of transplantation. *Transplantation* 37:434-437, 1984

150. Serie,JR, Hickey,GE, Schmitt,RV, Hegre,OD: Prolongation of culture-isolated neonatal islet xenografts without immunosuppression. *Transplantation* 36:6-11, 1983

151. Posselt,AM, Barker,CF, Tomaszewski,JE, Markmann,JF, Choti,MA, Naji,A: Induction of donor-specific unresponsiveness by intrathymic islet transplantation. *Science* 249:1293-1295, 1990

152. Rayat,GR, Korbutt,GS, Elliott,JF, Rajotte,RV: Survival and function of syngeneic rat islet grafts placed within the thymus versus under the kidney capsule. *Cell Transplant.* 6:597-602, 1997

153. Bobzien,B, Yasunami,Y, Majercik,M, Lacy,PE, Davie,JM: Intratesticular transplants of islet xenografts (rat to mouse). *Diabetes* 32:213-216, 1983

154. Tze,WJ, Tai,J: Successful intracerebral allotransplantation of pancreatic endocrine cells in spontaneous diabetic BB rats without immunosuppression. *Metabolism* 33:785-789, 1984

155. Salazar-Banuelos,A, Wright,JR, Jr., Sigalet,D, Benitez-Bribiesca,L: Pancreatic islet transplantation into the bone marrow of the rat. *Am.J.Surg.* 195:674-678, 2008

156. Salazar-Banuelos,A, Wright,J, Sigalet,D, Benitez-Bribiesca,L: The bone marrow as a potential receptor site for pancreatic islet grafts. *Arch.Med Res.* 39:139-141, 2008

157. Ryan,EA, Paty,BW, Senior,PA, Bigam,D, Alfadhli,E, Kneteman,NM, Lakey,JR, Shapiro,AM: Five-year follow-up after clinical islet transplantation. *Diabetes* 54:2060-2069, 2005

158. Bennet,W, Groth,CG, Larsson,R, Nilsson,B, Korsgren,O: Isolated human islets trigger an instant blood mediated inflammatory reaction: implications for intraportal islet transplantation as a treatment for patients with type 1 diabetes. *Ups.J.Med Sci.* 105:125-133, 2000

159. Contreras,JL: Extrahepatic transplant sites for islet xenotransplantation. *Xenotransplantation* 15:99-101, 2008

160. Ricordi,C, Finke,EH, Lacy,PE: A method for the mass isolation of islets from the adult pig pancreas. *Diabetes* 35:649-653, 1986

161. Lake,SP, Bassett,PD, Larkins,A, Revell,J, Walczak,K, Chamberlain,J, Rumford,GM, London,NJ, Veitch,PS, Bell,PR, .: Large-scale purification

of human islets utilizing discontinuous albumin gradient on IBM 2991 cell separator. *Diabetes* 38 Suppl 1:143-145, 1989

162. Warnock,GL, Kneteman,NM, Ryan,EA, Evans,MG, Seelis,RE, Halloran,PF, Rabinovitch,A, Rajotte,RV: Continued function of pancreatic islets after transplantation in type I diabetes. *Lancet* 2:570-572, 1989

163. Scharp,DW, Lacy,PE, Santiago,JV, McCullough,CS, Weide,LG, Falqui,L, Marchetti,P, Gingerich,RL, Jaffe,AS, Cryer,PE, .: Insulin independence after islet transplantation into type I diabetic patient. *Diabetes* 39:515-518, 1990

164. Shapiro,AM, Lakey,JR, Ryan,EA, Korbutt,GS, Toth,E, Warnock,GL, Kneteman,NM, Rajotte,RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N.Engl.J.Med* 343:230-238, 2000

165. Ryan,EA, Lakey,JR, Rajotte,RV, Korbutt,GS, Kin,T, Imes,S, Rabinovitch,A, Elliott,JF, Bigam,D, Kneteman,NM, Warnock,GL, Larsen,I, Shapiro,AM: Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes* 50:710-719, 2001

166. Ryan,EA, Lakey,JR, Paty,BW, Imes,S, Korbutt,GS, Kneteman,NM, Bigam,D, Rajotte,RV, Shapiro,AM: Successful islet transplantation: continued insulin reserve provides long-term glycemic control. *Diabetes* 51:2148-2157, 2002

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

168. Shapiro,AM, Lakey,JR, Paty,BW, Senior,PA, Bigam,DL, Ryan,EA: Strategic opportunities in clinical islet transplantation. *Transplantation* 79:1304-1307, 2005

169. Shapiro,AM, Ricordi,C, Hering,BJ, Auchincloss,H, Lindblad,R, Robertson,RP, Secchi,A, Brendel,MD, Berney,T, Brennan,DC, Cagliero,E, Alejandro,R, Ryan,EA, DiMercurio,B, Morel,P, Polonsky,KS, Reems,JA, Bretzel,RG, Bertuzzi,F, Froud,T, Kandaswamy,R, Sutherland,DE, Eisenbarth,G, Segal,M, Preiksaitis,J, Korbutt,GS, Barton,FB, Viviano,L, Seyfert-Margolis,V, Bluestone,J, Lakey,JR: International trial of the Edmonton protocol for islet transplantation. *N.Engl.J.Med* 355:1318-1330, 2006

170. Pileggi,A, Ricordi,C, Kenyon,NS, Froud,T, Baidal,DA, Kahn,A, Selvaggi,G, Alejandro,R: Twenty years of clinical islet transplantation at the Diabetes Research Institute--University of Miami. *Clin.Transpl.*177-204, 2004

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

172. Froud,T, Ricordi,C, Baidal,DA, Hafiz,MM, Ponte,G, Cure,P, Pileggi,A, Poggioli,R, Ichii,H, Khan,A, Ferreira,JV, Pugliese,A, Esquenazi,VV, Kenyon,NS, Alejandro,R: Islet transplantation in type 1 diabetes mellitus using cultured islets and steroid-free immunosuppression: Miami experience. *Am.J.Transplant.* 5:2037-2046, 2005

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

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

175. Ozmen,L, Ekdahl,KN, Elgue,G, Larsson,R, Korsgren,O, Nilsson,B: Inhibition of thrombin abrogates the instant blood-mediated inflammatory reaction triggered by isolated human islets: possible application of the thrombin inhibitor melagatran in clinical islet transplantation. *Diabetes* 51:1779-1784, 2002

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

177. Goto,M, Groth,CG, Nilsson,B, Korsgren,O: 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, 2004

178. Bhargava,R, Senior,PA, Ackerman,TE, Ryan,EA, Paty,BW, Lakey,JR, Shapiro,AM: Prevalence of hepatic steatosis after islet transplantation and its relation to graft function. *Diabetes* 53:1311-1317, 2004

179. Cabric,S, Sanchez,J, Lundgren,T, Foss,A, Felldin,M, Kallen,R, Salmela,K, Tibell,A, Tufveson,G, Larsson,R, Korsgren,O, Nilsson,B: Islet surface heparinization prevents the instant blood-mediated inflammatory reaction in islet transplantation. *Diabetes* 56:2008-2015, 2007

180. Moberg,L, Johansson,H, Lukinius,A, Berne,C, Foss,A, Kallen,R, Ostraat,O, Salmela,K, Tibell,A, Tufveson,G, Elgue,G, Nilsson,EK, Korsgren,O, Nilsson,B: Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. *Lancet* 360:2039-2045, 2002

181. Johansson, H. Acta Universitatis Upsaliensis Uppsala. 2007.

182. Tjernberg,J, Ekdahl,KN, Lambris,JD, Korsgren,O, Nilsson,B: Acute antibody-mediated complement activation mediates lysis of pancreatic islets cells and may cause tissue loss in clinical islet transplantation. *Transplantation* 85:1193-1199, 2008

183. d'Apice,AJ, Cowan,PJ: Gene-modified pigs. *Xenotransplantation* 15:87-90, 2008

184. van der Windt,DJ, Bottino,R, Casu,A, Campanile,N, Cooper,DK: Rapid loss of intraportally transplanted islets: an overview of pathophysiology and preventive strategies. *Xenotransplantation* 14:288-297, 2007

185. Fabian,MC, Lakey,JR, Rajotte,RV, Kneteman,NM: The efficacy and toxicity of rapamycin in murine islet transplantation. In vitro and in vivo studies. *Transplantation* 56:1137-1142, 1993

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

187. Pearson,T, Markees,TG, Serreze,DV, Pierce,MA, Wicker,LS, Peterson,LB, Shultz,LD, Mordes,JP, Rossini,AA, Greiner,DL: Islet cell autoimmunity and transplantation tolerance: two distinct mechanisms? *Ann.N.Y.Acad.Sci.* 1005:148-156, 2003

188. Okitsu,T, Bartlett,ST, Hadley,GA, Drachenberg,CB, Farney,AC: Recurrent autoimmunity accelerates destruction of minor and major histoincompatible islet grafts in nonobese diabetic (NOD) mice. *Am.J.Transplant.* 1:138-145, 2001

189. Shi,Q, Wang,D, Hadley,GA, Bingaman,AW, Bartlett,ST, Farber,DL: Long-term islet graft survival in NOD mice by abrogation of recurrent autoimmunity. *Diabetes* 53:2338-2345, 2004

190. Makhlouf,L, Grey,ST, Dong,V, Csizmadia,E, Arvelo,MB, Auchincloss,H, Jr., Ferran,C, Sayegh,MH: 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, 2004

191. Figliuzzi,M, Cornolti,R, Plati,T, Rajan,N, Adobati,F, Remuzzi,G, Remuzzi,A: Subcutaneous xenotransplantation of bovine pancreatic islets. *Biomaterials* 26:5640-5647, 2005

192. Lanza,RP, Kuhtreiber,WM, Ecker,D, Staruk,JE, Chick,WL: Xenotransplantation of porcine and bovine islets without

immunosuppression using uncoated alginate microspheres. *Transplantation* 59:1377-1384, 1995

193. Marchetti,P, Giannarelli,R, Cosimi,S, Masiello,P, Coppelli,A, Viacava,P, Navalesi,R: Massive isolation, morphological and functional characterization, and xenotransplantation of bovine pancreatic islets. *Diabetes* 44:375-381, 1995

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

195. Korbutt,GS, Elliott,JF, Ao,Z, Smith,DK, Warnock,GL, Rajotte,RV: Large scale isolation, growth, and function of porcine neonatal islet cells. *J.Clin.Invest* 97:2119-2129, 1996

196. Ricordi,C, Socci,C, Davalli,AM, Staudacher,C, Baro,P, Vertova,A, Sassi,I, Gavazzi,F, Pozza,G, Di,C, V: Isolation of the elusive pig islet. *Surgery* 107:688-694, 1990

197. Wright,JR, Jr., Pohajdak,B, Xu,BY, Leventhal,JR: Piscine islet xenotransplantation. *ILAR.J.* 45:314-323, 2004

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

199. Wright,JR, Jr., Polvi,S, MacLean,H: Experimental transplantation with principal islets of teleost fish (Brockmann bodies). Long-term function of tilapia islet tissue in diabetic nude mice. *Diabetes* 41:1528-1532, 1992

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

201. Rother,KI, Harlan,DM: Challenges facing islet transplantation for the treatment of type 1 diabetes mellitus. *J.Clin.Invest* 114:877-883, 2004

202. O'Connell,PJ: The rationale and practical issues for the maintenance of clean herds for clinical islet xenotransplantation. *Xenotransplantation* 15:91-92, 2008

203. Mellert,J, Hering,BJ, Liu,X, Brandhorst,D, Brandhorst,H, Federlin,K, Bretzel,RG, Hopt,UT: Intravenous glucose tolerance tests after porcine islet auto- and allotransplantation. *Transplant.Proc.* 29:2091-2092, 1997

204. Cardona,K, Milas,Z, Strobert,E, Cano,J, Jiang,W, Safley,SA, Gangappa,S, Hering,BJ, Weber,CJ, Pearson,TC, Larsen,CP: Engraftment of adult

porcine islet xenografts in diabetic nonhuman primates through targeting of costimulation pathways. *Am.J.Transplant.* 7:2260-2268, 2007

205. Cardona,K, Korbutt,GS, Milas,Z, Lyon,J, Cano,J, Jiang,W, Bello-Laborn,H, Hacquoil,B, Strobert,E, Gangappa,S, Weber,CJ, Pearson,TC, Rajotte,RV, Larsen,CP: Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat.Med* 12:304-306, 2006

206. Hering,BJ, Wijkstrom,M, Graham,ML, Hardstedt,M, Aasheim,TC, Jie,T, Ansite,JD, Nakano,M, Cheng,J, Li,W, Moran,K, Christians,U, Finnegan,C, Mills,CD, Sutherland,DE, Bansal-Pakala,P, Murtaugh,MP, Kirchhof,N, Schuurman,HJ: Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. *Nat.Med* 12:301-303, 2006

207. Sutherland,DE, Steffes,MW, Bauer,GE, McManus,D, Noe,BD, Najarian,JS: Isolation of human and porcine islets of Langerhans and islet transplantation in pigs. *J.Surg.Res.* 16:102-111, 1974

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

209. Kirchhof,N, Shibata,S, Wijkstrom,M, Kulick,DM, Salerno,CT, Clemmings,SM, Heremans,Y, Galili,U, Sutherland,DE, Dalmasso,AP, Hering,BJ: Reversal of diabetes in non-immunosuppressed rhesus macaques by intraportal porcine islet xenografts precedes acute cellular rejection. *Xenotransplantation* 11:396-407, 2004

210. Wennberg,L, Song,Z, Bennet,W, Zhang,J, Nava,S, Sundberg,B, Bari,S, Groth,CG, Korsgren,O: 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, 2001

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

212. Duvivier-Kali,VF, Omer,A, Lopez-Avalos,MD, O'Neil,JJ, Weir,GC: Survival of microencapsulated adult pig islets in mice in spite of an antibody response. *Am.J.Transplant.* 4:1991-2000, 2004

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

214. Rayat,GR, Rajotte,RV, Korbutt,GS: Potential application of neonatal porcine islets as treatment for type 1 diabetes: a review. *Ann.N.Y.Acad.Sci.* 875:175-188, 1999

215. Emamaullee,JA, Shapiro,AM, Rajotte,RV, Korbutt,G, Elliott,JF: Neonatal porcine islets exhibit natural resistance to hypoxia-induced apoptosis. *Transplantation* 82:945-952, 2006

216. Kirchhof,N, Hering,BJ, Geiss,V, Federlin,K, Bretzel,RG: Evidence for breed-dependent differences in porcine islets of Langerhans. *Transplant.Proc.* 26:616-617, 1994

217. Korbutt,GS: What type of islets should be used? *Xenotransplantation* 15:81-82, 2008

218. Prabhakaran,S, Hering,BJ: What strain of pig should be used? *Xenotransplantation* 15:83-86, 2008

219. Socci,C, Ricordi,C, Davalli,AM, Staudacher,C, Baro,P, Vertova,A, Freschi,M, Gavazzi,F, Braghi,S, Pozza,G, .: Selection of donors significantly improves pig islet isolation yield. *Horm.Metab Res.Suppl* 25:32-34, 1990

220. Bloch,K, Assa,S, Lazard,D, Abramov,N, Shalitin,S, Weintrob,N, Josefsberg,Z, Rapoport,M, Vardi,P: Neonatal pig islets induce a lower T-cell response than adult pig islets in IDDM patients. *Transplantation* 67:748-752, 1999

221. Appel,MC, Banuelos,SJ, Greiner,DL, Shultz,LD, Mordes,JP, Rossini,AA: Prolonged survival of neonatal porcine islet xenografts in mice treated with a donor-specific transfusion and anti-CD154 antibody. *Transplantation* 77:1341-1349, 2004

222. Binette,TM, Dufour,JM, Korbutt,GS: In vitro maturation of neonatal porcine islets: a novel model for the study of islet development and xenotransplantation. *Ann.N.Y.Acad.Sci.* 944:47-61, 2001

223. Vizzardelli,C, Molano,RD, Pileggi,A, Berney,T, Cattan,P, Fenjves,ES, Peel,A, Fraker,C, Ricordi,C, Inverardi,L: 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, 2002

224. Kobayashi,T, Harb,G, Rayat,GR: 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, 2005

225. Korbutt,GS, Ao,Z, Flashner,M, Rajotte,RV: 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, 1997

226. Rayat,GR, Rajotte,RV, Ao,Z, Korbutt,GS: 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, 2000

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

228. Valdes-Gonzalez,RA, Dorantes,LM, Garibay,GN, Bracho-Blanchet,E, Mendez,AJ, Davila-Perez,R, Elliott,RB, Teran,L, White,DJ: Xenotransplantation of porcine neonatal islets of Langerhans and Sertoli cells: a 4-year study. *Eur.J.Endocrinol.* 153:419-427, 2005

229. Street,CN, Lakey,JR, Seeberger,K, Helms,L, Rajotte,RV, Shapiro,AM, Korbutt,GS: Heterogenous expression of nestin in human pancreatic tissue precludes its use as an islet precursor marker. *J.Endocrinol.* 180:213-225, 2004

230. Rayat,GR, Rajotte,RV, Hering,BJ, Binette,TM, Korbutt,GS: In vitro and in vivo expression of Galalpha-(1,3)Gal on porcine islet cells is age dependent. *J.Endocrinol.* 177:127-135, 2003

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

232. Korsgren,O, Sandler,S, Landstrom,AS, Jansson,L, Andersson,A: Large-scale production of fetal porcine pancreatic isletlike cell clusters. An experimental tool for studies of islet cell differentiation and xenotransplantation. *Transplantation* 45:509-514, 1988

233. Sandler,S, Andersson,A, Schnell,A, Mellgren,A, Tollemar,J, Borg,H, Petersson,B, Groth,CG, Hellerstrom,C: Tissue culture of human fetal pancreas. Development and function of B-cells in vitro and transplantation of explants to nude mice. *Diabetes* 34:1113-1119, 1985

234. Lim,SM, Heng,KK, Poh,LH, Lim,NK, Seah,ML, Li,SQ: The development of fetal porcine islet cells as xenografts: preliminary observations. *Transplant.Proc.* 24:1519, 1992

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

236. Lukinius,A, Korsgren,O: 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, 2001

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

238. Bennet,W, Bjorkland,A, Sundberg,B, Davies,H, Liu,J, Holgersson,J, Korsgren,O: 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, 2000

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

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

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

242. Tree of Life Web Project, Eutheria. Placental Mammals. The Tree of Life Web Project . 1997.

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

244. Rayat,GR, Johnson,ZA, Beilke,JN, Korbutt,GS, Rajotte,RV, Gill,RG: The degree of phylogenetic disparity of islet grafts dictates the reliance on indirect CD4 T-cell antigen recognition for rejection. *Diabetes* 52:1433-1440, 2003

245. Gordon,EJ, Wicker,LS, Peterson,LB, Serreze,DV, Markees,TG, Shultz,LD, Rossini,AA, Greiner,DL, Mordes,JP: Autoimmune diabetes and resistance to xenograft transplantation tolerance in NOD mice. *Diabetes* 54:107-115, 2005

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

247. Bennet,W, Sundberg,B, Lundgren,T, Tibell,A, Groth,CG, Richards,A, White,DJ, Elgue,G, Larsson,R, Nilsson,B, Korsgren,O: Damage to porcine islets of Langerhans after exposure to human blood in vitro, or after intraportal transplantation to cynomologus monkeys: protective effects of sCR1 and heparin. *Transplantation* 69:711-719, 2000

248. Buhler,L, Deng,S, O'Neil,J, Kitamura,H, Koulmanda,M, Baldi,A, Rahier,J, Alwayn,IP, Appel,JZ, Awwad,M, Sachs,DH, Weir,G, Squifflet,JP, Cooper,DK, Morel,P: Adult porcine islet transplantation in baboons treated with conventional immunosuppression or a non-myeloablative regimen and CD154 blockade. *Xenotransplantation* 9:3-13, 2002

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

250. Rood,PP, Bottino,R, Balamurugan,AN, Smetanka,C, Ayares,D, Groth,CG, Murase,N, Cooper,DK, Trucco,M: Reduction of early graft loss after intraportal porcine islet transplantation in monkeys. *Transplantation* 83:202-210, 2007

251. Karlsson-Parra,A, Ridderstad,A, Wallgren,AC, Moller,E, Ljunggren,HG, Korsgren,O: Xenograft rejection of porcine islet-like cell clusters in normal and natural killer cell-depleted mice. *Transplantation* 61:1313-1320, 1996

252. Simeonovic,CJ, Townsend,MJ, Wilson,JD, McKenzie,KU, Ramsay,AJ, Matthaei,KI, Mann,DA, Young,IG: Eosinophils are not required for the rejection of neovascularized fetal pig proislet xenografts in mice. *J.Immunol.* 158:2490-2499, 1997

253. Wu,G, Korsgren,O, Zhang,J, Song,Z, Van Rooijen,N, Tibell,A: Pig islet xenograft rejection is markedly delayed in macrophage-depleted mice: a study in streptozotocin diabetic animals. *Xenotransplantation* 7:214-220, 2000

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

255. Omer,A, Keegan,M, Czismadia,E, De Vos,P, Van Rooijen,N, Bonner-Weir,S, Weir,GC: Macrophage depletion improves survival of porcine neonatal pancreatic cell clusters contained in alginate macrocapsules transplanted into rats. *Xenotransplantation* 10:240-251, 2003

256. Yi,S, Hawthorne,WJ, Lehnert,AM, Ha,H, Wong,JK, Van Rooijen,N, Davey,K, Patel,AT, Walters,SN, Chandra,A, O'Connell,PJ: T cell-activated macrophages are capable of both recognition and rejection of pancreatic islet xenografts. *J.Immunol.* 170:2750-2758, 2003

257. Ricordi,C, Lacy,PE, Sterbenz,K, Davie,JM: Low-temperature culture of human islets or in vivo treatment with L3T4 antibody produces a marked prolongation of islet human-to-mouse xenograft survival. *Proc.Natl.Acad.Sci.U.S.A* 84:8080-8084, 1987

258. Wilson,JD, Simeonovic,CJ, Ting,JH, Ceredig,R: Role of CD4+ T-lymphocytes in rejection by mice of fetal pig proislet xenografts. *Diabetes* 38 Suppl 1:217-219, 1989

259. Olack,BJ, Jaramillo,A, Benshoff,ND, Kaleem,Z, Swanson,CJ, Lowell,JA, Mohanakumar,T: Rejection of porcine islet xenografts mediated by CD4+ T cells activated through the indirect antigen recognition pathway. *Xenotransplantation* 9:393-401, 2002

260. Gill,RG, Wolf,L, Daniel,D, Coulombe,M: CD4+ T cells are both necessary and sufficient for islet xenograft rejection. *Transplant.Proc.* 26:1203, 1994

261. Gill,RG, Coulombe,M: Islet xenografting in autoimmune diabetes. *Transplant.Proc.* 26:1140, 1994

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

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

264. Grabstein,KH, Waldschmidt,TJ, Finkelman,FD, Hess,BW, Alpert,AR, Boiani,NE, Namen,AE, Morrissey,PJ: Inhibition of murine B and T lymphopoiesis in vivo by an anti-interleukin 7 monoclonal antibody. *J.Exp.Med.* 178:257-264, 1993

265. Rayat,GR, Gill,RG: Pancreatic islet xenotransplantation: barriers and prospects. *Curr.Diab.Rep.* 3:336-343, 2003

266. Lacy,PE: Present status of islet transplantation. *Clin.Invest Med* 10:496-498, 1987

267. Soderlund,J, Wennberg,L, Castanos-Velez,E, Biberfeld,P, Zhu,S, Tibell,A, Groth,CG, Korsgren,O: Fetal porcine islet-like cell clusters transplanted to cynomolgus monkeys: an immunohistochemical study. *Transplantation* 67:784-791, 1999

268. Rajotte,RV: Moving towards clinical application. *Xenotransplantation* 15:113-115, 2008

269. Schuurman,HJ: Regulatory aspects of pig-to-human islet transplantation. *Xenotransplantation* 15:116-120, 2008

270. Boneva,RS, Folks,TM: Xenotransplantation and risks of zoonotic infections. *Ann.Med.* 36:504-517, 2004

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

272. Patience,C, Takeuchi,Y, Weiss,RA: Infection of human cells by an endogenous retrovirus of pigs. *Nat.Med* 3:282-286, 1997

273. Wilson,CA, Wong,S, Muller,J, Davidson,CE, Rose,TM, Burd,P: Type C retrovirus released from porcine primary peripheral blood mononuclear cells infects human cells. *J.Virol.* 72:3082-3087, 1998

274. Schmidt,P, Forsman,A, Andersson,G, Blomberg,J, Korsgren,O: Pig islet xenotransplantation: activation of porcine endogenous retrovirus in the immediate post-transplantation period. *Xenotransplantation* 12:450-456, 2005

275. van der Laan,LJ, Lockey,C, Griffeth,BC, Frasier,FS, Wilson,CA, Onions,DE, Hering,BJ, Long,Z, Otto,E, Torbett,BE, Salomon,DR: Infection by porcine endogenous retrovirus after islet xenotransplantation in SCID mice. *Nature* 407:90-94, 2000

276. Paradis,K, Langford,G, Long,Z, Heneine,W, Sandstrom,P, Switzer,WM, Chapman,LE, Lockey,C, Onions,D, Otto,E: Search for cross-species transmission of porcine endogenous retrovirus in patients treated with living pig tissue. The XEN 111 Study Group. *Science* 285:1236-1241, 1999

277. Elliott,RB, Escobar,L, Garkavenko,O, Croxson,MC, Schroeder,BA, McGregor,M, Ferguson,G, Beckman,N, Ferguson,S: No evidence of

infection with porcine endogenous retrovirus in recipients of encapsulated porcine islet xenografts. *Cell Transplant.* 9:895-901, 2000

278. Heneine,W, Tibell,A, Switzer,WM, Sandstrom,P, Rosales,GV, Mathews,A, Korsgren,O, Chapman,LE, Folks,TM, Groth,CG: No evidence of infection with porcine endogenous retrovirus in recipients of porcine islet-cell xenografts. *Lancet* 352:695-699, 1998

279. Nanji,SA, Shapiro,AM: Islet transplantation in patients with diabetes mellitus: choice of immunosuppression. *BioDrugs.* 18:315-328, 2004

280. Borel,JF, Baumann,G, Chapman,I, Donatsch,P, Fahr,A, Mueller,EA, Vigouret,JM: In vivo pharmacological effects of ciclosporin and some analogues. *Adv.Pharmacol.* 35:115-246, 1996

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

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

283. Gunnarsson,R, Klintmalm,G, Lundgren,G, Tyden,G, Wilczek,H, Ostman,J, Groth,CG: Deterioration in glucose metabolism in pancreatic transplant recipients after conversion from azathioprine to cyclosporine. *Transplant.Proc.* 16:709-712, 1984

284. Meier-Kriesche,HU, Li,S, Gruessner,RW, Fung,JJ, Bustami,RT, Barr,ML, Leichtman,AB: Immunosuppression: evolution in practice and trends, 1994-2004. *Am.J.Transplant.* 6:1111-1131, 2006

285. Jindal,RM: Posttransplant diabetes mellitus--a review. *Transplantation* 58:1289-1298, 1994

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

287. Rilo,HL, Carroll,PB, Zeng,YJ, Fontes,P, Demetris,J, Ricordi,C: Acceleration of chronic failure of intrahepatic canine islet autografts by a short course of prednisone. *Transplantation* 57:181-187, 1994

288. Zeng,Y, Ricordi,C, Lendoire,J, Carroll,PB, Alejandro,R, Bereiter,DR, Tzakis,A, Starzl,TE: The effect of prednisone on pancreatic islet autografts in dogs. *Surgery* 113:98-102, 1993

289. Nanji,SA, Shapiro,AM: Advances in pancreatic islet transplantation in humans. *Diabetes Obes.Metab* 8:15-25, 2006

290. Abraham,RT, Wiederrecht,GJ: Immunopharmacology of rapamycin. *Annu.Rev.Immunol.* 14:483-510, 1996

291. Vezina,C, Kudelski,A, Sehgal,SN: 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, 1975

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

293. Dumont,FJ, Su,Q: Mechanism of action of the immunosuppressant rapamycin. *Life Sci.* 58:373-395, 1996

294. Rothstein,DM, Sayegh,MH: T-cell costimulatory pathways in allograft rejection and tolerance. *Immunol.Rev.* 196:85-108, 2003

295. Clarkson,MR, Sayegh,MH: T-cell costimulatory pathways in allograft rejection and tolerance. *Transplantation* 80:555-563, 2005

296. Sayegh,MH, Turka,LA: The role of T-cell costimulatory activation pathways in transplant rejection. *N.Engl.J.Med* 338:1813-1821, 1998

297. Jenkins,MK, Taylor,PS, Norton,SD, Urdahl,KB: CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J.Immunol.* 147:2461-2466, 1991

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

299. Kroczek,RA, Mages,HW, Hutloff,A: Emerging paradigms of T-cell co-stimulation. *Curr.Opin.Immunol.* 16:321-327, 2004

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

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

302. Bielekova,B, Catalfamo,M, Reichert-Scrivner,S, Packer,A, Cerna,M, Waldmann,TA, McFarland,H, Henkart,PA, Martin,R: 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, 2006

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

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

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

306. Calne,R, Moffatt,SD, Friend,PJ, Jamieson,NV, Bradley,JA, Hale,G, Firth,J, Bradley,J, Smith,KG, Waldmann,H: Campath IH allows low-dose cyclosporine monotherapy in 31 cadaveric renal allograft recipients. *Transplantation* 68:1613-1616, 1999

307. Knechtle,SJ, Pirsch,JD, Fechner,H, Becker,BN, Friedl,A, Colvin,RB, Lebeck,LK, Chin,LT, Becker,YT, Odorico,JS, D'Alessandro,AM, Kalayoglu,M, Hamawy,MM, Hu,H, Bloom,DD, Sollinger,HW: Campath-1H induction plus rapamycin monotherapy for renal transplantation: results of a pilot study. *Am.J.Transplant.* 3:722-730, 2003

308. Rao,V, Pirsch,JD, Becker,BN, Knechtle,SJ: Sirolimus monotherapy following Campath-1H induction. *Transplant.Proc.* 35:128S-130S, 2003

309. Hering,BJ, Kandaswamy,R, Harmon,JV, Ansite,JD, Clemmings,SM, Sakai,T, Paraskevas,S, Eckman,PM, Sageshima,J, Nakano,M, Sawada,T, Matsumoto,I, Zhang,HJ, Sutherland,DE, Bluestone,JA: Transplantation of cultured islets from two-layer preserved pancreases in type 1 diabetes with anti-CD3 antibody. *Am.J.Transplant.* 4:390-401, 2004

310. Gribben,JG, Guinan,EC, Boussiotis,VA, Ke,XY, Linsley,L, Sieff,C, Gray,GS, Freeman,GJ, Nadler,LM: Complete blockade of B7 family-mediated costimulation is necessary to induce human alloantigen-specific anergy: a method to ameliorate graft-versus-host disease and extend the donor pool. *Blood* 87:4887-4893, 1996

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

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

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

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

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

316. Lenschow,DJ, Zeng,Y, Thistlethwaite,JR, Montag,A, Brady,W, Gibson,MG, Linsley,PS, Bluestone,JA: Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science* 257:789-792, 1992

317. Safley,SA, Kapp,JA, Weber,CJ: Proliferative and cytokine responses in CTLA4-Ig-treated diabetic NOD mice transplanted with microencapsulated neonatal porcine ICCs. *Cell Transplant.* 11:695-705, 2002

318. Larsen,CP, Pearson,TC, Adams,AB, Tso,P, Shirasugi,N, Strobertm,E, Anderson,D, Cowan,S, Price,K, Naemura,J, Emswiler,J, Greene,J, Turk,LA, Bajorath,J, Townsend,R, Hagerty,D, Linsley,PS, Peach,RJ: Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am.J.Transplant.* 5:443-453, 2005

319. Vincenti,F, Larsen,C, Durrbach,A, Wekerle,T, Nashan,B, Blancho,G, Lang,P, Grinyo,J, Halloran,PF, Solez,K, Hagerty,D, Levy,E, Zhou,W, Natarajan,K, Charpentier,B: Costimulation blockade with belatacept in renal transplantation. *N.Engl.J.Med* 353:770-781, 2005

320. Xu,Y, Song,G: The role of CD40-CD154 interaction in cell immunoregulation. *J.Biomed.Sci.* 11:426-438, 2004

321. Henn,V, Slupsky,JR, Grafe,M, Anagnostopoulos,I, Forster,R, Muller-Berghaus,G, Kroczek,RA: CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 391:591-594, 1998

322. Kirk,AD, Blair,PJ, Tadaki,DK, Xu,H, Harlan,DM: The role of CD154 in organ transplant rejection and acceptance. *Philos.Trans.R.Soc.Lond B Biol.Sci.* 356:691-702, 2001

323. Quezada,SA, Jarvinen,LZ, Lind,EF, Noelle,RJ: CD40/CD154 interactions at the interface of tolerance and immunity. *Annu.Rev.Immunol.* 22:307-328, 2004

324. Caux,C, Massacrier,C, Vanbervliet,B, Dubois,B, Van Kooten,C, Durand,I, Banchereau,J: Activation of human dendritic cells through CD40 cross-linking. *J.Exp.Med* 180:1263-1272, 1994

325. Kiener,PA, Moran-Davis,P, Rankin,BM, Wahl,AF, Aruffo,A, Hollenbaugh,D: Stimulation of CD40 with purified soluble gp39 induces proinflammatory responses in human monocytes. *J.Immunol.* 155:4917-4925, 1995

326. Bleharski,JR, Niazi,KR, Sieling,PA, Cheng,G, Modlin,RL: Signaling lymphocytic activation molecule is expressed on CD40 ligand-activated dendritic cells and directly augments production of inflammatory cytokines. *J.Immunol.* 167:3174-3181, 2001

327. Gao,W, Demirci,G, Strom,TB, Li,XC: Stimulating PD-1-negative signals concurrent with blocking CD154 co-stimulation induces long-term islet allograft survival. *Transplantation* 76:994-999, 2003

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

329. Benda,B, Ljunggren,HG, Peach,R, Sandberg,JO, Korsgren,O: Co-stimulatory molecules in islet xenotransplantation: CTLA4Ig treatment in CD40 ligand-deficient mice. *Cell Transplant.* 11:715-720, 2002

330. Lehnert,AM, Yi,S, Burgess,JS, O'Connell,PJ: 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, 2000

331. Kenyon,NS, Fernandez,LA, Lehmann,R, Masetti,M, Ranuncoli,A, Chatzipetrou,M, Iaria,G, Han,D, Wagner,JL, Ruiz,P, Berho,M, Inverardi,L, Alejandro,R, Mintz,DH, Kirk,AD, Harlan,DM, Burkly,LC, Ricordi,C: Long-term survival and function of intrahepatic islet allografts in baboons treated with humanized anti-CD154. *Diabetes* 48:1473-1481, 1999

332. Kenyon,NS, Chatzipetrou,M, Masetti,M, Ranuncoli,A, Oliveira,M, Wagner,JL, Kirk,AD, Harlan,DM, Burkly,LC, Ricordi,C: Long-term survival and function of intrahepatic islet allografts in rhesus monkeys treated with humanized anti-CD154. *Proc.Natl.Acad.Sci.U.S.A* 96:8132-8137, 1999

333. Kawai,T, Andrews,D, Colvin,RB, Sachs,DH, Cosimi,AB: Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat.Med* 6:114, 2000

334. Yamada,AA, Sayegh,MH: The CD154-CD40 costimulatory pathway in transplantation. *Transplantation* 73:S36-S39, 2002

335. Kover,KL, Geng,Z, Hess,DM, Benjamin,CD, Moore,WV: Anti-CD154 (CD40L) prevents recurrence of diabetes in islet isografts in the DR-BB rat. *Diabetes* 49:1666-1670, 2000

336. Molano,RD, Berney,T, Li,H, Cattan,P, Pileggi,A, Vizzardelli,C, Kenyon,NS, Ricordi,C, Burkly,LC, Inverardi,L: Prolonged islet graft survival in NOD mice by blockade of the CD40-CD154 pathway of T-cell costimulation. *Diabetes* 50:270-276, 2001

337. Hancock,WW: Chemokine receptor-dependent alloresponses. *Immunol.Rev.* 196:37-50, 2003

338. Yopp,AC, Fu,S, Honig,SM, Randolph,GJ, Ding,Y, Krieger,NR, Bromberg,JS: FTY720-enhanced T cell homing is dependent on CCR2, CCR5, CCR7, and CXCR4: evidence for distinct chemokine compartments. *J.Immunol.* 173:855-865, 2004

339. Akashi,S, Sho,M, Kashizuka,H, Hamada,K, Ikeda,N, Kuzumoto,Y, Tsurui,Y, Nomi,T, Mizuno,T, Kanehiro,H, Hisanaga,M, Ko,S, Nakajima,Y: A novel small-molecule compound targeting CCR5 and CXCR3 prevents acute and chronic allograft rejection. *Transplantation* 80:378-384, 2005

340. Lee,I, Wang,L, Wells,AD, Ye,Q, Han,R, Dorf,ME, Kuziel,WA, Rollins,BJ, Chen,L, Hancock,WW: Blocking the monocyte chemoattractant protein-1/CCR2 chemokine pathway induces permanent survival of islet allografts through a programmed death-1 ligand-1-dependent mechanism. *J.Immunol.* 171:6929-6935, 2003

341. Wijkstrom,M, Kenyon,NS, Kirchhof,N, Kenyon,NM, Mullon,C, Lake,P, Cottens,S, Ricordi,C, Hering,BJ: Islet allograft survival in nonhuman primates immunosuppressed with basiliximab, RAD, and FTY720. *Transplantation* 77:827-835, 2004

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

343. Godwin,JW, d'Apice,AJ, Cowan,PJ: Characterization of pig intercellular adhesion molecule-2 and its interaction with human LFA-1. *Am.J.Transplant.* 4:515-525, 2004

344. Davignon,D, Martz,E, Reynolds,T, Kurzinger,K, Springer,TA: 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, 1981

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

346. Milicevic,NM, Nohroudi,K, Milicevic,Z, Westermann,J: Blood lymphocytes, monocytes and NK cells modulate their expression of CD44, ICAM-1, LFA-1 and MHC class II after arrival into lymphoid organs. *Immunol.Invest* 33:439-452, 2004

347. Van Epps,DE, Potter,J, Vachula,M, Smith,CW, Anderson,DC: Suppression of human lymphocyte chemotaxis and transendothelial migration by anti-LFA-1 antibody. *J.Immunol.* 143:3207-3210, 1989

348. Go,S, Fleischmann,A, Lantz,O, Cretolle,C, Brousse,N, Cerf-Bensussan,N, Sarnacki,S: Anti-LFA-1 antibody postpones T-cell receptor triggering while preserving generation of regulatory T cells in T-cell receptor anti-HY transgenic mice. *Transplantation* 82:119-126, 2006

349. Ostermann,G, Weber,KS, Zernecke,A, Schroder,A, Weber,C: JAM-1 is a ligand of the beta(2) integrin LFA-1 involved in transendothelial migration of leukocytes. *Nat.Immunol.* 3:151-158, 2002

350. Shamri,R, Grabovsky,V, Gauguet,JM, Feigelson,S, Manevich,E, Kolanus,W, Robinson,MK, Staunton,DE, von Andrian,UH, Alon,R: Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nat.Immunol.* 6:497-506, 2005

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

352. Borthwick,NJ, Akbar,AA, Buckley,C, Pilling,D, Salmon,M, Jewell,AP, Yong,KL: Transendothelial migration confers a survival advantage to activated T lymphocytes: role of LFA-1/ICAM-1 interactions. *Clin.Exp.Immunol.* 134:246-252, 2003

353. Chesnutt,BC, Smith,DF, Raffler,NA, Smith,ML, White,EJ, Ley,K: Induction of LFA-1-dependent neutrophil rolling on ICAM-1 by engagement of E-selectin. *Microcirculation.* 13:99-109, 2006

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

355. Jun,CD, Shimaoka,M, Carman,CV, Takagi,J, Springer,TA: Dimerization and the effectiveness of ICAM-1 in mediating LFA-1-dependent adhesion. *Proc.Natl.Acad.Sci.U.S.A* 98:6830-6835, 2001

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

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

358. Ni,HT, Deeths,MJ, Li,W, Mueller,DL, Mescher,MF: Signaling pathways activated by leukocyte function-associated Ag-1-dependent costimulation. *J.Immunol.* 162:5183-5189, 1999

359. Perez,OD, Mitchell,D, Jager,GC, South,S, Murriel,C, McBride,J, Herzenberg,LA, Kinoshita,S, Nolan,GP: Leukocyte functional antigen 1 lowers T cell activation thresholds and signaling through cytohesin-1 and Jun-activating binding protein 1. *Nat.Immunol.* 4:1083-1092, 2003

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

361. Nicolls,MR, Gill,RG: LFA-1 (CD11a) as a therapeutic target. *Am.J.Transplant.* 6:27-36, 2006

362. Abraham,C, Griffith,J, Miller,J: The dependence for leukocyte function-associated antigen-1/ICAM-1 interactions in T cell activation cannot be overcome by expression of high density TCR ligand. *J.Immunol.* 162:4399-4405, 1999

363. Grakoui,A, Bromley,SK, Sumen,C, Davis,MM, Shaw,AS, Allen,PM, Dustin,ML: The immunological synapse: a molecular machine controlling T cell activation. *Science* 285:221-227, 1999

364. Dustin,ML, Bivona,TG, Philips,MR: Membranes as messengers in T cell adhesion signaling. *Nat.Immunol.* 5:363-372, 2004

365. Bianchi,E, Denti,S, Granata,A, Bossi,G, Geginat,J, Villa,A, Rogge,L, Pardi,R: Integrin LFA-1 interacts with the transcriptional co-activator JAB1 to modulate AP-1 activity. *Nature* 404:617-621, 2000

366. Mor,A, Campi,G, Du,G, Zheng,Y, Foster,DA, Dustin,ML, Philips,MR: The lymphocyte function-associated antigen-1 receptor costimulates plasma membrane Ras via phospholipase D2. *Nat.Cell Biol.* 9:713-719, 2007

367. Barber,DF, Faure,M, Long,EO: LFA-1 contributes an early signal for NK cell cytotoxicity. *J.Immunol.* 173:3653-3659, 2004

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

369. Larsson,LC, Corbascio,M, Widner,H, Pearson,TC, Larsen,CP, Ekberg,H: Simultaneous inhibition of B7 and LFA-1 signaling prevents rejection of discordant neural xenografts in mice lacking CD40L. *Xenotransplantation.* 9:68-76, 2002

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

371. Nicolls,MR, Coulombe,M, Beilke,J, Gelhaus,HC, Gill,RG: CD4-dependent generation of dominant transplantation tolerance induced by simultaneous perturbation of CD154 and LFA-1 pathways. *J.Immunol.* 169:4831-4839, 2002

372. Tredget,EB, Arefanian,H, Gill,RG, Rajotte,RV, Rayat,GR: Monotherapy with anti-LFA-1 monoclonal antibody promotes long-term survival of rat islet xenografts. *Cell Transplant.* 17:599-608, 2008

373. Lebwohl,M, Tyring,SK, Hamilton,TK, Toth,D, Glazer,S, Tawfik,NH, Walicke,P, Dummer,W, Wang,X, Garovoy,MR, Pariser,D: A novel targeted T-cell modulator, efalizumab, for plaque psoriasis. *N.Engl.J.Med.* 349:2004-2013, 2003

374. Anderson,CC: 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, 2006

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

376. Lanza,RP, Hayes,JL, Chick,WL: Encapsulated cell technology. *Nat.Biotechnol.* 14:1107-1111, 1996

377. Lanza,RP, Chick,WL: Transplantation of encapsulated cells and tissues. *Surgery* 121:1-9, 1997

378. Maki,T, Otsu,I, O'Neil,JJ, Dunleavy,K, Mullon,CJ, Solomon,BA, Monaco,AP: Treatment of diabetes by xenogeneic islets without immunosuppression. Use of a vascularized bioartificial pancreas. *Diabetes* 45:342-347, 1996

379. Lanza,RP, Chick,WL: Immunoisolation: at a turning point. *Immunol.Today* 18:135-139, 1997

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

381. Isayeva,IS, Kasibhatla,BT, Rosenthal,KS, Kennedy,JP: Characterization and performance of membranes designed for macroencapsulation/implantation of pancreatic islet cells. *Biomaterials* 24:3483-3491, 2003

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

383. La Flamme,KE, Mor,G, Gong,D, La Tempa,T, Fusaro,VA, Grimes,CA, Desai,TA: Nanoporous alumina capsules for cellular macroencapsulation: transport and biocompatibility. *Diabetes Technol.Ther.* 7:684-694, 2005

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

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

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

387. De Vos,P, Wolters,GH, Fritschy,WM, Van Schilfgaarde,R: Obstacles in the application of microencapsulation in islet transplantation. *Int.J.Artif.Organs* 16:205-212, 1993

388. Orive,G, Gascon,AR, Hernandez,RM, Igartua,M, Luis,PJ: Cell microencapsulation technology for biomedical purposes: novel insights and challenges. *Trends Pharmacol.Sci.* 24:207-210, 2003

389. Orive,G, Hernandez,RM, Rodriguez,GA, Calafiore,R, Chang,TM, De Vos,P, Hortelano,G, Hunkeler,D, Lacik,I, Pedraz,JL: History, challenges and perspectives of cell microencapsulation. *Trends Biotechnol.* 22:87-92, 2004

390. Korbutt,GS, Elliott,JF, Rajotte,RV: Cotransplantation of allogeneic islets with allogeneic testicular cell aggregates allows long-term graft survival without systemic immunosuppression. *Diabetes* 46:317-322, 1997

391. Dickson,BC, Yang,H, Savelkoul,HF, Rowden,G, Van Rooijen,N, Wright,JR, Jr.: 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, 2003

392. Duvivier-Kali,VF, Omer,A, Parent,RJ, O'Neil,JJ, Weir,GC: Complete protection of islets against allorejection and autoimmunity by a simple barium-alginate membrane. *Diabetes* 50:1698-1705, 2001

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

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

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

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

397. Fritschy,WM, Wolters,GH, Van Schilfgaarde,R: Effect of alginate-polylysine-alginate microencapsulation on in vitro insulin release from rat pancreatic islets. *Diabetes* 40:37-43, 1991

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

399. Selawry,HP, Cameron,DF: Sertoli cell-enriched fractions in successful islet cell transplantation. *Cell Transplant.* 2:123-129, 1993

400. Sipione,S, Simmen,KC, Lord,SJ, Motyka,B, Ewen,C, Shostak,I, Rayat,GR, Dufour,JM, Korbutt,GS, Rajotte,RV, Bleackley,RC: Identification of a novel human granzyme B inhibitor secreted by cultured sertoli cells. *J.Immunol.* 177:5051-5058, 2006

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

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

403. Griffith,TS, Brunner,T, Fletcher,SM, Green,DR, Ferguson,TA: Fas ligand-induced apoptosis as a mechanism of immune privilege. *Science* 270:1189-1192, 1995

404. Jenne,DE, Tschopp,J: 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, 1989

405. Merly,F, Huard,C, Asselin,I, Robbins,PD, Tremblay,JP: Anti-inflammatory effect of transforming growth factor-beta1 in myoblast transplantation. *Transplantation* 65:793-799, 1998

406. Wahl,SM, McCartney-Francis,N, Mergenhagen,SE: Inflammatory and immunomodulatory roles of TGF-beta. *Immunol.Today* 10:258-261, 1989

407. Sykes,M: Immune tolerance: mechanisms and application in clinical transplantation. *J.Intern.Med* 262:288-310, 2007

408. BILLINGHAM,RE, BRENT,L, MEDAWAR,PB: Actively acquired tolerance of foreign cells. *Nature* 172:603-606, 1953

409. Owen,RD: IMMUNOGENETIC CONSEQUENCES OF VASCULAR ANASTOMOSES BETWEEN BOVINE TWINS. *Science* 102:400-401, 1945

410. Hasek,M: Vegetative hybridization of animals by joint blood circulation during embryonal development. 1953. *Transplantation* 76:1412-1421, 2003

411. BRENT,L: The 50th anniversary of the discovery of immunologic tolerance. *N.Engl.J.Med* 349:1381-1383, 2003

412. BRENT,L: Commentary on the "birth" of immunologic tolerance half a century ago. *Transplantation* 76:1423-1424, 2003

413. Burnet, F. M. and and F.Fenner. The Production of Antibodies. Macmillan and Company Limited, Melbourne, Australia. 1949.
Ref Type: Report

414. Palmer,E: Negative selection--clearing out the bad apples from the T-cell repertoire. *Nat.Rev.Immunol.* 3:383-391, 2003

415. Liston,A, Lesage,S, Wilson,J, Peltonen,L, Goodnow,CC: Aire regulates negative selection of organ-specific T cells. *Nat.Immunol.* 4:350-354, 2003

416. Anderson,MS, Venanzi,ES, Klein,L, Chen,Z, Berzins,SP, Turley,SJ, von Boehmer,H, Bronson,R, Dierich,A, Benoist,C, Mathis,D: Projection of an immunological self shadow within the thymus by the aire protein. *Science* 298:1395-1401, 2002

417. Derbinski,J, Schulte,A, Kyewski,B, Klein,L: Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat.Immunol.* 2:1032-1039, 2001

418. Nagamine,K, Peterson,P, Scott,HS, Kudoh,J, Minoshima,S, Heino,M, Krohn,KJ, Lalioti,MD, Mullis,PE, Antonarakis,SE, Kawasaki,K, Asakawa,S, Ito,F, Shimizu,N: Positional cloning of the APECED gene. *Nat.Genet.* 17:393-398, 1997

419. Gillard,GO, Farr,AG: Features of medullary thymic epithelium implicate postnatal development in maintaining epithelial heterogeneity and tissue-restricted antigen expression. *J.Immunol.* 176:5815-5824, 2006

420. Mathis,D, Benoist,C: A decade of AIRE. *Nat.Rev.Immunol.* 7:645-650, 2007

421. Gebe,JA, Falk,BA, Rock,KA, Kochik,SA, Heninger,AK, Reijonen,H, Kwok,WW, Nepom,GT: Low-avidity recognition by CD4+ T cells directed to self-antigens. *Eur.J.Immunol.* 33:1409-1417, 2003

422. Rider,BJ, Fraga,E, Yu,Q, Singh,B: Immune responses to self peptides naturally presented by murine class II major histocompatibility complex molecules. *Mol.Immunol.* 33:625-633, 1996

423. Ridge,JP, Di Rosa,F, Matzinger,P: A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* 393:474-478, 1998

424. Kurts,C, Carbone,FR, Barnden,M, Blanas,E, Allison,J, Heath,WR, Miller,JF: CD4+ T cell help impairs CD8+ T cell deletion induced by cross-presentation of self-antigens and favors autoimmunity. *J.Exp.Med* 186:2057-2062, 1997

425. Aversa,G, Cocks,BG, Punnonen,J, Carballido,JM, de Vries,JE: Contact-mediated signals and cytokines involved in B-cell activation and isotype switching in pre-B and mature B cells. *Res.Immunol.* 145:222-226, 1994

426. Foy,TM, Shepherd,DM, Durie,FH, Aruffo,A, Ledbetter,JA, Noelle,RJ: In vivo CD40-gp39 interactions are essential for thymus-dependent humoral

immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39. *J.Exp.Med* 178:1567-1575, 1993

427. Stockinger,B, Grant,CF, Hausmann,B: Localization of self antigen: implications for antigen presentation and induction of tolerance. *Eur.J.Immunol.* 23:6-11, 1993

428. Morrison,LA, Lukacher,AE, Braciale,VL, Fan,DP, Braciale,TJ: Differences in antigen presentation to MHC class I-and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. *J.Exp.Med* 163:903-921, 1986

429. Cosimi,AB, Sachs,DH: Mixed chimerism and transplantation tolerance. *Transplantation* 77:943-946, 2004

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

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

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

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

434. Baker,MB, Altman,NH, Podack,ER, Levy,RB: The role of cell-mediated cytotoxicity in acute GVHD after MHC-matched allogeneic bone marrow transplantation in mice. *J.Exp.Med.* 183:2645-2656, 1996

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

436. Wysocki,CA, Panoskaltsis-Mortari,A, Blazar,BR, Serody,JS: Leukocyte migration and graft-versus-host disease. *Blood* 105:4191-4199, 2005

437. Gallegos,AM, Bevan,MJ: Central tolerance: good but imperfect. *Immunol.Rev.* 209:290-296, 2006

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

439. Levy,MM, Ketchum,RJ, Tomaszewski,JE, Naji,A, Barker,CF, Brayman,KL: 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, 2002

440. Oluwole,OO, Depaz,HA, Gopinathan,R, Ali,A, Garrovillo,M, Jin,MX, Hardy,MA, Oluwole,SF: 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, 2001

441. Turvey,SE, Hara,M, Morris,PJ, Wood,KJ: Mechanisms of tolerance induction after intrathymic islet injection: determination of the fate of alloreactive thymocytes. *Transplantation* 68:30-39, 1999

442. Ali,A, Garrovillo,M, Jin,MX, Hardy,MA, Oluwole,SF: Major histocompatibility complex class I peptide-pulsed host dendritic cells induce antigen-specific acquired thymic tolerance to islet cells. *Transplantation* 69:221-226, 2000

443. Saborio,DV, Chowdhury,NC, Jin,MX, Chandraker,A, Sayegh,MH, Oluwole,SF: Regulatory T cells maintain peripheral tolerance to islet allografts induced by intrathymic injection of MHC class I allopeptides. *Cell Transplant.* 8:375-381, 1999

444. Wu,A, Yamada,K, Neville,DM, Awwad,M, Wain,JC, Shimizu,A, Gojo,S, Kitamura,H, Colvin,RB, Cooper,DK, Sykes,M, Sachs,DH: Xenogeneic thymus transplantation in a pig-to-baboon model. *Transplantation* 75:282-291, 2003

445. Lee,LA, Gritsch,HA, Sergio,JJ, Arn,JS, Glaser,RM, Sablinski,T, Sachs,DH, Sykes,M: Specific tolerance across a discordant xenogeneic transplantation barrier. *Proc.Natl.Acad.Sci.U.S.A* 91:10864-10867, 1994

446. Yamamoto,S, Lavelle,JM, Vagefi,PA, Arakawa,H, Samelson-Jones,E, Moran,S, Teranishi,K, Kamano,C, Fishman,J, Awwad,M, Neville,DM, Shimizu,A, Sykes,M, Sachs,DH, Yamada,K: 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, 2005

447. Rodriguez-Barbosa,JI, Zhao,Y, Zhao,G, Ezquerra,A, Sykes,M: 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, 2002

448. Larsen,CP, Elwood,ET, Alexander,DZ, Ritchie,SC, Hendrix,R, Tucker-Burden,C, Cho,HR, Aruffo,A, Hollenbaugh,D, Linsley,PS, Winn,KJ, Pearson,TC: Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature* 381:434-438, 1996

449. Larsen,CP, Pearson,TC: The CD40 pathway in allograft rejection, acceptance, and tolerance. *Curr.Opin.Immunol.* 9:641-647, 1997

450. Lu,LF, Lind,EF, Gondek,DC, Bennett,KA, Gleeson,MW, Pino-Lagos,K, Scott,ZA, Coyle,AJ, Reed,JL, Van Snick,J, Strom,TB, Zheng,XX, Noelle,RJ: Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature* 442:997-1002, 2006

451. Larsen,CP, Alexander,DZ, Hollenbaugh,D, Elwood,ET, Ritchie,SC, Aruffo,A, Hendrix,R, Pearson,TC: CD40-gp39 interactions play a critical role during allograft rejection. Suppression of allograft rejection by blockade of the CD40-gp39 pathway. *Transplantation* 61:4-9, 1996

452. Quezada,SA, Bennett,K, Blazar,BR, Rudensky,AY, Sakaguchi,S, Noelle,RJ: Analysis of the underlying cellular mechanisms of anti-CD154-induced graft tolerance: the interplay of clonal anergy and immune regulation. *J.Immunol.* 175:771-779, 2005

453. Kirk,AD, Harlan,DM, Armstrong,NN, Davis,TA, Dong,Y, Gray,GS, Hong,X, Thomas,D, Fechner,JH, Jr., Knechtle,SJ: CTLA4-Ig and anti-CD40 ligand prevent renal allograft rejection in primates. *Proc.Natl.Acad.Sci.U.S.A* 94:8789-8794, 1997

454. Kirk,AD, Burkly,LC, Batty,DS, Baumgartner,RE, Berning,JD, Buchanan,K, Fechner,JH, Jr., Germond,RL, Kampen,RL, Patterson,NB, Swanson,SJ, Tadaki,DK, TenHoor,CN, White,L, Knechtle,SJ, Harlan,DM: Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates. *Nat.Med* 5:686-693, 1999

455. Kirk,AD, Tadaki,DK, Celniker,A, Batty,DS, Berning,JD, Colonna,JO, Cruzata,F, Elster,EA, Gray,GS, Kampen,RL, Patterson,NB, Szklut,P, Swanson,J, Xu,H, Harlan,DM: Induction therapy with monoclonal antibodies specific for CD80 and CD86 delays the onset of acute renal allograft rejection in non-human primates. *Transplantation* 72:377-384, 2001

456. Gett,AV, Hodgkin,PD: A cellular calculus for signal integration by T cells. *Nat.Immunol.* 1:239-244, 2000

457. Sinclair,NR, Chan,PL: Relationship between antibody-mediated immunosuppression and tolerance induction. *Nature* 234:104-105, 1971

458. Anderson,CC, Sinclair,NR: FcR-mediated inhibition of cell activation and other forms of coinhibition. *Crit Rev.Immunol.* 18:525-544, 1998

459. Sinclair,NR, Anderson,CC: Co-stimulation and co-inhibition: equal partners in regulation. *Scand.J.Immunol.* 43:597-603, 1996

460. Sinclair,NR: How many signals are enough? *Cell Immunol.* 130:204-212, 1990

461. Okazaki,T, Tanaka,Y, Nishio,R, Mitsuiye,T, Mizoguchi,A, Wang,J, Ishida,M, Hiai,H, Matsumori,A, Minato,N, Honjo,T: Autoantibodies against cardiac troponin I are responsible for dilated cardiomyopathy in PD-1-deficient mice. *Nat.Med* 9:1477-1483, 2003

462. Sabatos,CA, Chakravarti,S, Cha,E, Schubart,A, Sanchez-Fueyo,A, Zheng,XX, Coyle,AJ, Strom,TB, Freeman,GJ, Kuchroo,VK: Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. *Nat.Immunol.* 4:1102-1110, 2003

463. Sanchez-Fueyo,A, Tian,J, Picarella,D, Domenig,C, Zheng,XX, Sabatos,CA, Manlongat,N, Bender,O, Kamradt,T, Kuchroo,VK, Gutierrez-Ramos,JC, Coyle,AJ, Strom,TB: Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. *Nat.Immunol.* 4:1093-1101, 2003

464. Perez,VL, Van Parijs,L, Biuckians,A, Zheng,XX, Strom,TB, Abbas,AK: Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity.* 6:411-417, 1997

465. Caspi,RR, Chan,CC, Grubbs,BG, Silver,PB, Wiggert,B, Parsa,CF, Bahmanyar,S, Billiau,A, Heremans,H: Endogenous systemic IFN-gamma has a protective role against ocular autoimmunity in mice. *J.Immunol.* 152:890-899, 1994

466. Ravetch,JV, Lanier,LL: Immune inhibitory receptors. *Science* 290:84-89, 2000

467. Sinclair,NR: Why so many coinhibitory receptors? *Scand.J.Immunol.* 50:10-13, 1999

468. Wekerle,T, Sykes,M: Mixed chimerism as an approach for the induction of transplantation tolerance. *Transplantation* 68:459-467, 1999

469. Alam,SM, Travers,PJ, Wung,JL, Nasholds,W, Redpath,S, Jameson,SC, Gascoigne,NR: T-cell-receptor affinity and thymocyte positive selection. *Nature* 381:616-620, 1996

470. Allen,PM: Peptides in positive and negative selection: a delicate balance. *Cell* 76:593-596, 1994

471. Baldwin,TA, Hogquist,KA, Jameson,SC: The fourth way? Harnessing aggressive tendencies in the thymus. *J.Immunol.* 173:6515-6520, 2004

472. Inaba,M, Inaba,K, Hosono,M, Kumamoto,T, Ishida,T, Muramatsu,S, Masuda,T, Ikehara,S: Distinct mechanisms of neonatal tolerance induced by dendritic cells and thymic B cells. *J.Exp.Med* 173:549-559, 1991

473. Ramsdell,F, Fowlkes,BJ: Clonal deletion versus clonal anergy: the role of the thymus in inducing self tolerance. *Science* 248:1342-1348, 1990

474. Ferber,I, Schonrich,G, Schenkel,J, Mellor,AL, Hammerling,GJ, Arnold,B: Levels of peripheral T cell tolerance induced by different doses of tolerogen. *Science* 263:674-676, 1994

475. Heath,WR, Kurts,C, Miller,JF, Carbone,FR: Cross-tolerance: a pathway for inducing tolerance to peripheral tissue antigens. *J.Exp.Med* 187:1549-1553, 1998

476. Moskophidis,D, Lechner,F, Pircher,H, Zinkernagel,RM: Virus persistence in acutely infected immunocompetent mice by exhaustion of antiviral cytotoxic effector T cells. *Nature* 362:758-761, 1993

477. Wekerle,T, Kurtz,J, Ito,H, Ronquillo,JV, Dong,V, Zhao,G, Shaffer,J, Sayegh,MH, Sykes,M: Allogeneic bone marrow transplantation with co-stimulatory blockade induces macrochimerism and tolerance without cytoreductive host treatment. *Nat.Med* 6:464-469, 2000

478. Wekerle,T, Sayegh,MH, Hill,J, Zhao,Y, Chandraker,A, Swenson,KG, Zhao,G, Sykes,M: Extrathymic T cell deletion and allogeneic stem cell engraftment induced with costimulatory blockade is followed by central T cell tolerance. *J.Exp.Med* 187:2037-2044, 1998

479. Iwakoshi,NN, Markees,TG, Turgeon,N, Thornley,T, Cuthbert,A, Leif,J, Phillips,NE, Mordes,JP, Greiner,DL, Rossini,AA: Skin allograft maintenance in a new synchimeric model system of tolerance. *J.Immunol.* 167:6623-6630, 2001

480. Wells,AD, Li,XC, Li,Y, Walsh,MC, Zheng,XX, Wu,Z, Nunez,G, Tang,A, Sayegh,M, Hancock,WW, Strom,TB, Turka,LA: Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat.Med* 5:1303-1307, 1999

481. Zhang,ZX, Yang,L, Young,KJ, DuTemple,B, Zhang,L: Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nat.Med* 6:782-789, 2000

482. Appleman,LJ, Boussiotis,VA: T cell anergy and costimulation. *Immunol.Rev.* 192:161-180, 2003

483. Rutella,S, Danese,S, Leone,G: Tolerogenic dendritic cells: cytokine modulation comes of age. *Blood* 108:1435-1440, 2006

484. Hoves,S, Krause,SW, Schutz,C, Halbritter,D, Scholmerich,J, Herfarth,H, Fleck,M: Monocyte-derived human macrophages mediate anergy in allogeneic T cells and induce regulatory T cells. *J.Immunol.* 177:2691-2698, 2006

485. Chiodetti,L, Choi,S, Barber,DL, Schwartz,RH: Adaptive tolerance and clonal anergy are distinct biochemical states. *J.Immunol.* 176:2279-2291, 2006

486. Schwartz,RH: Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy. *Cell* 71:1065-1068, 1992

487. Wells,AD, Walsh,MC, Bluestone,JA, Turka,LA: Signaling through CD28 and CTLA-4 controls two distinct forms of T cell anergy. *J.Clin.Invest* 108:895-903, 2001

488. Kurtz,J, Shaffer,J, Lie,A, Anosova,N, Benichou,G, Sykes,M: Mechanisms of early peripheral CD4 T-cell tolerance induction by anti-CD154 monoclonal antibody and allogeneic bone marrow transplantation: evidence for anergy and deletion but not regulatory cells. *Blood* 103:4336-4343, 2004

489. Arnold,B, Schonrich,G, Hammerling,GJ: Multiple levels of peripheral tolerance. *Immunol.Today* 14:12-14, 1993

490. Rocken,M, Urban,JF, Shevach,EM: Infection breaks T-cell tolerance. *Nature* 359:79-82, 1992

491. Knoechel,B, Lohr,J, Zhu,S, Wong,L, Hu,D, Ausubel,L, Abbas,AK: Functional and molecular comparison of anergic and regulatory T lymphocytes. *J.Immunol.* 176:6473-6483, 2006

492. Rocha,B, Grandien,A, Freitas,AA: Anergy and exhaustion are independent mechanisms of peripheral T cell tolerance. *J.Exp.Med* 181:993-1003, 1995

493. Chai,JG, Bartok,I, Scott,D, Dyson,J, Lechler,R: T:T antigen presentation by activated murine CD8+ T cells induces anergy and apoptosis. *J.Immunol.* 160:3655-3665, 1998

494. Vendetti,S, Chai,JG, Dyson,J, Simpson,E, Lombardi,G, Lechler,R: Anergic T cells inhibit the antigen-presenting function of dendritic cells. *J.Immunol.* 165:1175-1181, 2000

495. Vanasek,TL, Nandiwada,SL, Jenkins,MK, Mueller,DL: CD25+Foxp3+ regulatory T cells facilitate CD4+ T cell clonal anergy induction during the recovery from lymphopenia. *J.Immunol.* 176:5880-5889, 2006

496. Li,L, Godfrey,WR, Porter,SB, Ge,Y, June,CH, Blazar,BR, Boussiotis,VA: CD4+CD25+ regulatory T-cell lines from human cord blood have functional and molecular properties of T-cell anergy. *Blood* 106:3068-3073, 2005

497. Goodnow,CC: Transgenic mice and analysis of B-cell tolerance. *Annu.Rev.Immunol.* 10:489-518, 1992

498. Ochsenbein,AF, Sierro,S, Odermatt,B, Pericin,M, Karrer,U, Hermans,J, Hemmi,S, Hengartner,H, Zinkernagel,RM: Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction. *Nature* 411:1058-1064, 2001

499. Heath,WR, Allison,J, Hoffmann,MW, Schonrich,G, Hammerling,G, Arnold,B, Miller,JF: Autoimmune diabetes as a consequence of locally produced interleukin-2. *Nature* 359:547-549, 1992

500. von Herrath,MG, Guerder,S, Lewicki,H, Flavell,RA, Oldstone,MB: Coexpression of B7-1 and viral ("self") transgenes in pancreatic beta cells can break peripheral ignorance and lead to spontaneous autoimmune diabetes. *Immunity.* 3:727-738, 1995

501. Ohashi,PS, Oehen,S, Buerki,K, Pircher,H, Ohashi,CT, Odermatt,B, Malissen,B, Zinkernagel,RM, Hengartner,H: Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell* 65:305-317, 1991

502. Lafferty,KJ, Babcock,SK, Gill,RG: Prevention of rejection by treatment of the graft: an overview. *Prog.Clin.Biol.Res.* 224:87-117, 1986

503. Sakaguchi,S, Sakaguchi,N, Asano,M, Itoh,M, Toda,M: Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J.Immunol.* 155:1151-1164, 1995

504. Groux,H, Powrie,F: Regulatory T cells and inflammatory bowel disease. *Immunol.Today* 20:442-445, 1999

505. Battaglia,M, Stabilini,A, Draghici,E, Gregori,S, Mocchetti,C, Bonifacio,E, Roncarolo,MG: Rapamycin and interleukin-10 treatment induces T

regulatory type 1 cells that mediate antigen-specific transplantation tolerance. *Diabetes* 55:40-49, 2006

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

507. Ikemoto,T, Tashiro,S, Yasutomo,K, Kishihara,K, Kurita,N, Miyake,H: 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, 2004

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

509. Sanchez-Fueyo,A, Weber,M, Domenig,C, Strom,TB, Zheng,XX: Tracking the immunoregulatory mechanisms active during allograft tolerance. *J.Immunol.* 168:2274-2281, 2002

510. Honey,K, Cobbold,SP, Waldmann,H: CD40 ligand blockade induces CD4+ T cell tolerance and linked suppression. *J.Immunol.* 163:4805-4810, 1999

511. Graca,L, Honey,K, Adams,E, Cobbold,SP, Waldmann,H: Cutting edge: anti-CD154 therapeutic antibodies induce infectious transplantation tolerance. *J.Immunol.* 165:4783-4786, 2000

512. Graca,L, Cobbold,SP, Waldmann,H: Identification of regulatory T cells in tolerated allografts. *J.Exp.Med* 195:1641-1646, 2002

513. Graca,L, Thompson,S, Lin,CY, Adams,E, Cobbold,SP, Waldmann,H: Both CD4(+)CD25(+) and CD4(+)CD25(-) regulatory cells mediate dominant transplantation tolerance. *J.Immunol.* 168:5558-5565, 2002

514. Cobbold,SP, Castejon,R, Adams,E, Zelenika,D, Graca,L, Humm,S, Waldmann,H: Induction of foxP3+ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *J.Immunol.* 172:6003-6010, 2004

515. Sykes M, Auchincloss Jr H, and , Sachs DH. Transplantation immunology. In: Paul WE, ed. Fundamental Immunology. Philadelphia, PA: Lippincott-Raven. 1481-1555. 2003.

516. Hall,BM, Pearce,NW, Gurley,KE, Dorsch,SE: Specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with

cyclosporine. III. Further characterization of the CD4+ suppressor cell and its mechanisms of action. *J.Exp.Med* 171:141-157, 1990

517. Hara,M, Kingsley,CI, Niimi,M, Read,S, Turvey,SE, Bushell,AR, Morris,PJ, Powrie,F, Wood,KJ: IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J.Immunol.* 166:3789-3796, 2001

518. Zhai,Y, Meng,L, Gao,F, Wang,Y, Busuttil,RW, Kupiec-Weglinski,JW: CD4+ T regulatory cell induction and function in transplant recipients after CD154 blockade is TLR4 independent. *J.Immunol.* 176:5988-5994, 2006

519. Kitade,H, Kawai,M, Rutgeerts,O, Landuyt,W, Waer,M, Mathieu,C, Pirenne,J: Early presence of regulatory cells in transplanted rats rendered tolerant by donor-specific blood transfusion. *J.Immunol.* 175:4963-4970, 2005

520. Bushell,A, Karim,M, Kingsley,CI, Wood,KJ: Pretransplant blood transfusion without additional immunotherapy generates CD25+CD4+ regulatory T cells: a potential explanation for the blood-transfusion effect. *Transplantation* 76:449-455, 2003

521. Karim,M, Bushell,AR, Wood,KJ: Regulatory T cells in transplantation. *Curr.Opin.Immunol.* 14:584-591, 2002

522. Zhai,Y, Kupiec-Weglinski,JW: What is the role of regulatory T cells in transplantation tolerance? *Curr.Opin.Immunol.* 11:497-503, 1999

523. Karim,M, Kingsley,CI, Bushell,AR, Sawitzki,BS, Wood,KJ: Alloantigen-induced CD25+CD4+ regulatory T cells can develop in vivo from CD25-CD4+ precursors in a thymus-independent process. *J.Immunol.* 172:923-928, 2004

524. Tran,HM, Nickerson,PW, Restifo,AC, Ivis-Woodward,MA, Patel,A, Allen,RD, Strom,TB, O'Connell,PJ: Distinct mechanisms for the induction and maintenance of allograft tolerance with CTLA4-Fc treatment. *J.Immunol.* 159:2232-2239, 1997

525. Porter,CM, Bloom,ET: Human CD4+CD25+ regulatory T cells suppress anti-porcine xenogeneic responses. *Am.J.Transplant.* 5:2052-2057, 2005

526. Fontenot,JD, Gavin,MA, Rudensky,AY: Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat.Immunol.* 4:330-336, 2003

527. Hori,S, Nomura,T, Sakaguchi,S: Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061, 2003

528. O'Garra,A, Vieira,P: Regulatory T cells and mechanisms of immune system control. *Nat.Med* 10:801-805, 2004

529. Huang,X, Zhu,J, Yang,Y: Protection against autoimmunity in nonlymphopenic hosts by CD4+ CD25+ regulatory T cells is antigen-specific and requires IL-10 and TGF-beta. *J.Immunol.* 175:4283-4291, 2005

530. Huber,S, Schramm,C, Lehr,HA, Mann,A, Schmitt,S, Becker,C, Protschka,M, Galle,PR, Neurath,MF, Blessing,M: Cutting edge: TGF-beta signaling is required for the in vivo expansion and immunosuppressive capacity of regulatory CD4+CD25+ T cells. *J.Immunol.* 173:6526-6531, 2004

531. Marie,JC, Letterio,JJ, Gavin,M, Rudensky,AY: TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *J.Exp.Med* 201:1061-1067, 2005

532. Karim,M, Feng,G, Wood,KJ, Bushell,AR: CD25+CD4+ regulatory T cells generated by exposure to a model protein antigen prevent allograft rejection: antigen-specific reactivation in vivo is critical for bystander regulation. *Blood* 105:4871-4877, 2005

533. Thornton,AM, Shevach,EM: Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J.Immunol.* 164:183-190, 2000

534. Sawitzki,B, Kingsley,CI, Oliveira,V, Karim,M, Herber,M, Wood,KJ: IFN-gamma production by alloantigen-reactive regulatory T cells is important for their regulatory function in vivo. *J.Exp.Med* 201:1925-1935, 2005

535. Malek,TR, Bayer,AL: Tolerance, not immunity, crucially depends on IL-2. *Nat.Rev.Immunol.* 4:665-674, 2004

536. Fu,S, Zhang,N, Yopp,AC, Chen,D, Mao,M, Chen,D, Zhang,H, Ding,Y, Bromberg,JS: TGF-beta induces Foxp3 + T-regulatory cells from CD4 + C. *Am.J.Transplant.* 4:1614-1627, 2004

537. Roncarolo,MG, Gregori,S, Battaglia,M, Bacchetta,R, Fleischhauer,K, Levings,MK: Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol.Rev.* 212:28-50, 2006

538. Jonuleit,H, Schmitt,E, Schuler,G, Knop,J, Enk,AH: Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J.Exp.Med* 192:1213-1222, 2000

539. Li,MO, Sanjabi,S, Flavell,RA: Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity.* 25:455-471, 2006

540. Zheng,SG, Wang,JH, Stohl,W, Kim,KS, Gray,JD, Horwitz,DA: TGF-beta requires CTLA-4 early after T cell activation to induce FoxP3 and generate adaptive CD4+CD25+ regulatory cells. *J.Immunol.* 176:3321-3329, 2006

541. Marie,JC, Liggitt,D, Rudensky,AY: Cellular mechanisms of fatal early-onset autoimmunity in mice with the T cell-specific targeting of transforming growth factor-beta receptor. *Immunity.* 25:441-454, 2006

542. Battaglia,M, Roncarolo,MG: Induction of transplantation tolerance via regulatory T cells. *Inflamm.Allergy Drug Targets.* 5:157-165, 2006

543. Wood,KJ, Sakaguchi,S: Regulatory T cells in transplantation tolerance. *Nat.Rev.Immunol.* 3:199-210, 2003

544. Bacchetta,R, Passerini,L, Gambineri,E, Dai,M, Allan,SE, Perroni,L, Dagna-Bricarelli,F, Sartirana,C, Matthes-Martin,S, Lawitschka,A, Azzari,C, Ziegler,SF, Levings,MK, Roncarolo,MG: Defective regulatory and effector T cell functions in patients with FOXP3 mutations. *J.Clin.Invest* 116:1713-1722, 2006

545. Cohen,AC, Nadeau,KC, Tu,W, Hwa,V, Dionis,K, Bezrodnik,L, Teper,A, Gaillard,M, Heinrich,J, Krensky,AM, Rosenfeld,RG, Lewis,DB: Cutting edge: Decreased accumulation and regulatory function of CD4+ CD25(high) T cells in human STAT5b deficiency. *J.Immunol.* 177:2770-2774, 2006

546. Zorn,E, Nelson,EA, Mohseni,M, Porcheray,F, Kim,H, Litsa,D, Bellucci,R, Raderschall,E, Canning,C, Soiffer,RJ, Frank,DA, Ritz,J: IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. *Blood* 108:1571-1579, 2006

547. Mosmann,TR, Coffman,RL: TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu.Rev.Immunol.* 7:145-173, 1989

548. Croft,M, Carter,L, Swain,SL, Dutton,RW: Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. *J.Exp.Med* 180:1715-1728, 1994

549. Le Moine,A, Goldman,M, Abramowicz,D: Multiple pathways to allograft rejection. *Transplantation* 73:1373-1381, 2002

550. Higuchi,M, Zeng,D, Shizuru,J, Gworek,J, Dejbakhsh-Jones,S, Taniguchi,M, Strober,S: Immune tolerance to combined organ and bone marrow transplants after fractionated lymphoid irradiation involves regulatory NK T cells and clonal deletion. *J.Immunol.* 169:5564-5570, 2002

551. Sykes,M, Hoyles,KA, Romick,ML, Sachs,DH: In vitro and in vivo analysis of bone marrow-derived CD3+, CD4-, CD8-, NK1.1+ cell lines. *Cell Immunol.* 129:478-493, 1990

552. Zeng,D, Lewis,D, Dejbakhsh-Jones,S, Lan,F, Garcia-Ojeda,M, Sibley,R, Strober,S: Bone marrow NK1.1(-) and NK1.1(+) T cells reciprocally regulate acute graft versus host disease. *J.Exp.Med* 189:1073-1081, 1999

553. Ciubotariu,R, Colovai,AI, Pennesi,G, Liu,Z, Smith,D, Berlocco,P, Cortesini,R, Suciu-Foca,N: Specific suppression of human CD4+ Th cell responses to pig MHC antigens by CD8+. *J.Immunol.* 161:5193-5202, 1998

554. Faunce,DE, Terajewicz,A, Stein-Streilein,J: Cutting edge: in vitro-generated tolerogenic APC induce CD8+ T regulatory cells that can suppress ongoing experimental autoimmune encephalomyelitis. *J.Immunol.* 172:1991-1995, 2004

555. Liu,J, Liu,Z, Witkowski,P, Vlad,G, Manavalan,JS, Scotto,L, Kim-Schulze,S, Cortesini,R, Hardy,MA, Suciu-Foca,N: Rat CD8+ FOXP3+ T suppressor cells mediate tolerance to allogeneic heart transplants, inducing PIR-B in APC and rendering the graft invulnerable to rejection. *Transpl.Immunol.* 13:239-247, 2004

556. Hoglund,P: Induced peripheral regulatory T cells: the family grows larger. *Eur.J.Immunol.* 36:264-266, 2006

557. Noble,A, Giorgini,A, Leggat,JA: Cytokine-induced IL-10-secreting CD8 T cells represent a phenotypically distinct suppressor T-cell lineage. *Blood* 107:4475-4483, 2006

558. Xia,G, Kovochich,M, Truitt,RL, Johnson,BD: Tracking ex vivo-expanded CD4+CD25+ and CD8+CD25+ regulatory T cells after infusion to prevent donor lymphocyte infusion-induced lethal acute graft-versus-host disease. *Biol.Blood Marrow Transplant.* 10:748-760, 2004

559. Xystrakis,E, Dejean,AS, Bernard,I, Druet,P, Liblau,R, Gonzalez-Dunia,D, Saoudi,A: Identification of a novel natural regulatory CD8 T-cell subset and analysis of its mechanism of regulation. *Blood* 104:3294-3301, 2004

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

561. Myers,L, Takahashi,C, Mittler,RS, Rossi,RJ, Vella,AT: Effector CD8 T cells possess suppressor function after 4-1BB and Toll-like receptor triggering. *Proc.Natl.Acad.Sci.U.S.A* 100:5348-5353, 2003

562. Laffont,S, Coudert,JD, Garidou,L, Delpy,L, Wiedemann,A, Demur,C, Coureau,C, Guery,JC: CD8+ T-cell-mediated killing of donor dendritic cells prevents alloreactive T helper type-2 responses in vivo. *Blood* 108:2257-2264, 2006

563. Miller,RG, Muraoka,S, Claesson,MH, Reimann,J, Benveniste,P: The veto phenomenon in T-cell regulation. *Ann.N.Y.Acad.Sci.* 532:170-176, 1988

564. Verbanac,KM, Carver,FM, Haisch,CE, Thomas,JM: A role for transforming growth factor-beta in the veto mechanism in transplant tolerance. *Transplantation* 57:893-900, 1994

565. Godfrey,WR, Spoden,DJ, Ge,YG, Baker,SR, Liu,B, Levine,BL, June,CH, Blazar,BR, Porter,SB: Cord blood CD4(+)CD25(+)-derived T regulatory cell lines express FoxP3 protein and manifest potent suppressor function. *Blood* 105:750-758, 2005

566. Tang,Q, Henriksen,KJ, Bi,M, Finger,EB, Szot,G, Ye,J, Masteller,EL, McDevitt,H, Bonyhadi,M, Bluestone,JA: In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J.Exp.Med* 199:1455-1465, 2004

CHAPTER 2

SHORT-TERM ADMINISTRATIONS OF A COMBINATION OF ANTI-LFA-1 AND ANTI-CD154 MONOCLONAL ANTIBODIES INDUCES TOLERANCE TO NEONATAL PORCINE ISLET XENOGRAFTS

A version of this chapter has been submitted for publication. Arefanian, H, et al. *Diabetes* 10.2337/DB09-0413: 2009

2.1 INTRODUCTION

Islet transplantation at present is a treatment alternative for a very select patient population, and is not available for children with type 1 diabetes partly due to the requirement for continuous use of harmful immunosuppressive drugs to prevent rejection of the islet grafts, and the severe shortage of human donor pancreatic tissue (1-3). Although immunosuppressive drugs have been instrumental in controlling the immune responses to islet grafts, their harmful side effects such as infection, malignancy and drug-specific toxicities leave patients at risk (2-7). Moreover, the shortage of human donor pancreatic tissue is further compounded by the requirement of typically two or more donor pancreases to completely free patients from exogenous insulin injection (8-12).

Islets from neonatal pigs are being considered for clinical transplantation because they are easy to isolate and maintain in culture as well as abundant number of islets can be isolated from one neonatal pig pancreas (13). In addition, neonatal porcine islets (NPI) have the inherent ability to proliferate and differentiate as well as they are capable of reversing diabetes in both small (13-15) and large animals (16;17), including the pre-clinical non-human primate model (17). A promising strategy that we previously found to be highly effective in preventing NPI xenograft rejection in mice is the combination of biologic agents in the form of anti-LFA-1 and anti-CD154 monoclonal antibodies (mAbs) (14). We demonstrated that short-term administrations of a combination of anti-LFA-1 and anti-CD154 mAbs promote long-term survival of NPI xenografts in B6 mice (14;15) and in spontaneously diabetic NOD mice (18). These studies

suggest that both adhesion and co-stimulatory pathways of T cell activation are important components of NPI xenograft rejection and that simultaneous targeting of these pathways is beneficial for long-term survival of NPI xenografts. The aim of this study was to determine whether interference of adhesion and co-stimulatory pathways by transient administrations of a combination of anti-LFA-1 and anti-CD154 mAbs could induce tolerance to phylogenetically disparate NPI xenografts in mice. Our results show for the first time that short-term administrations of combined anti-LFA-1 and anti-CD154 mAbs result in a robust form of pig islet xenograft tolerance mediated by T regulatory cells in B6 mice.

2.2 MATERIALS AND METHODS

2.2.1 Animals

One to 3-day-old Duroc cross-neonatal pigs (>1.5 kg body weight) from the University of Alberta (Edmonton, Alberta, Canada) were used as islet donors. Six to 8 week-old male B6 (C57BL/6J, H-2^b) and B6 *rag-/-* (B6.129S7-$Rag1^{tm1Mom}$/J, H-2^b) mice from Jackson Laboratory (Bar Harbor, ME, USA) were used as recipients of islet transplants. These mice were rendered diabetic by a single intraperitoneal injection of 180 or 175 mg/kg body weight of streptozotocin (STZ, Sigma, St Louis, MO, USA) for B6 or B6 *rag-/-* mice, respectively. Blood glucose levels of these mice were measured using a Precision glucose meter (ONETOUCH, Ultra, Lifescan, Milpitas, CA, USA). All mice were fed standard laboratory food and cared for according to the guidelines established by the Canadian Council on Animal Care.

2.2.2 Islet isolation and transplantation

NPI were isolated as described previously (13;14;18). Briefly, neonatal pigs were anesthetized with halothane and subjected to laparotomy and exsanguination. The pancreas was removed, placed in Hanks' balanced salt solution (HBSS, Sigma, St Louis, MO, USA) cut into small pieces, and digested with 2.5 mg/ml collagenase (clostridiopeptidase A, type XI, Sigma-Aldrich, St. Louis, MO, USA). Digested tissue was filtered through a 500 μm nylon screen then cultured for 7 days in HAM's F10 medium (GIBCO Laboratories, Grand Island, NY, USA) containing 10 mmol/l glucose, 50 μmol/l isobutylmethylxanthine (ICN Biomedicals, Montreal, Canada), 0.5% bovine serum albumin (fraction V, radioimmunoassay grade; Sigma), 2 mmol/l L-glutamine, 3 mmol/l $CaCl_2$, 10 mmol/l nicotinamide (BDH Biochemical, Poole, England), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C (5% CO_2, 95% air). After 7 days of culture, NPI were counted for transplantation and a total of 2,000 NPI were transplanted under the left kidney capsule of B6 or B6 *rag-/-* mice that had two consecutive non-fasting blood glucose levels of >20 mmol/l 4 to 6 days after STZ injection (13;14). Briefly, diabetic B6 or B6 *rag-/-* mice were anesthetized by inhalational isoflurane and the left flank was shaved and sterilized with 100% ethanol. A flank incision was made lateral to the left paraspinal muscles and the peritoneum was sharply divided to expose the left kidney. A sterile cotton swab was utilized to expose and produce the left kidney from the incision. A small incision was made in the kidney capsule with a 27-gauge needle and a subcapsular pocket was expanded with the use of a sterile

Pasteur pipette. Aliquots of 2,000 NPI were aspirated into polyethylene (PE-50) tubing, pelleted by centrifugation and placed within the subcapsular pocket with the aid of a micromanipulator syringe. Once the tubing was removed, the kidney capsule was cauterized with a disposable high-temperature cautery pen (Aaron Medical Industries, St. Petersburg, FL, USA). Islet engraftment was considered successful when blood glucose levels of these mice reached ≤8.5 mmol/l. Graft rejection was defined as the first of three consecutive days of hyperglycemia (>12 mmol/l), and was confirmed by histological analysis of the graft. Removal of the graft-bearing kidney was performed in recipients with long-term graft function (>150 days post-transplantation) to confirm that maintenance of normoglycemia was due to the presence of islet xenograft.

2.2.3 Monoclonal antibody therapies

B6 mouse recipients of NPI were randomly designated to receive short-term administration of a combination of anti-LFA-1 mAb (KBA; rat IgG2a, prepared as ascites; 200 μg on days 0, 1, 7, and 14 post-transplantation) plus anti-CD154 mAb (MR-1, hamster IgG, BioExpress, West Lebanon, NH, USA; 250 μg on days –1, 1 and 2 times a week for 4 weeks post-transplantation) intraperitoneally (i.p.). Some of the B6 mouse recipients of NPI with ling-term islet graft survival received i.p. injection of anti-CD25 mAb (PC6.1.5.3, rat IgG1, Bioexpress; 500 μg on 0, 2, 4 and 6 days post-administration starting at 150 days post-transplantation).

2.2.4 Oral glucose tolerance test

At 150 days post-transplantation, an oral glucose tolerance test (OGTT) was performed in randomly selected B6 and B6 *rag-/-* mice transplanted with NPI that maintained long-term graft function as well as in naïve B6 mice (control group) following our previously described method (13). Briefly mice were initially deprived of food for 16 hours and were given 50% solution of D-glucose (3 mg/g body weight) using an oral gavage. Glucose levels were measured using a Precision glucose meter (ONETOUCH, Ultra, Lifescan) in blood samples obtained from the tail vein of mice before oral administration of glucose and at 15, 30, 60, 90 and 120 min after oral glucose administration**.**

2.2.5 Immunohistological analysis

The presence of insulin-producing beta cells and immune cell infiltrate in the graft were examined by fixing one-half of the graft-bearing kidneys in 10% buffered formalin solution and embedding the tissue in paraffin. Five 5 μm sections of the tissues were stained with guinea pig anti-porcine insulin primary antibody (1:1000 dilutions; DAKO laboratories, Mississauga, Ontario, Canada) for 30 min, followed by the addition of biotinylated goat anti-guinea pig IgG secondary antibody (1:200 dilutions; Vector Laboratories, Burlingame, CA, USA). Avidin-biotin complex/horseradish peroxidase (ABC/HP; Vector Laboratories, Burlingame, CA, USA) and 3, 3-diaminobenzidinetetrahydrochloride (DAB; BioGenex, San Ramon, CA, USA) were used to detect cell stained positive for insulin (brown color). All paraffin-embedded sections were counter-stained with Harris' hematoxylin and eosin. The other half of the kidney was embedded in

OCT compound (Miles Scientific, Naperville, IL, USA) and kept frozen at –80°C to identify the foxp3 positive cells in the grafts. Five-micron frozen sections were air dried for 10 min, fixed in acetone for 3 min at 4°C, incubated in 0.1% Triton X-100 in PBS for 10 min, and then washed in PBS. Non-specific binding was eliminated by incubating the tissue sections in 2% fetal bovine serum (FBS) in PBS for 30 min. In addition, endogenous avidin and biotin or biotin-binding proteins present in the sections were also eliminated using the avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, USA). Rat anti-mouse foxp3 antibody (1:25 dilutions; eBioscience, San Diego, CA, USA) was applied to designated tissue sections for 60 min at room temperature. Biotinylated goat anti-rat IgG (1:200 dilutions; Southern Biotechnology Associates, Inc., Birmingham, AL, USA) secondary antibody was added and incubated for 30 min. ABC/HP reagent (Vector Laboratories) and DAB were applied to produce a brown color. Sections were then counter-stained with Harris' hematoxylin (14;18).

2.2.6 RNA extraction and RT-PCR analysis

Islet grafts and surrounding kidney parenchyma were collected using sterile instruments treated overnight with RNAse Away (Molecular Bio-Products, San Diego, CA, USA). Naïve B6 mouse kidney and thymus tissue was also collected as control tissue for RNA extraction and subsequent RT-PCR analysis. Tissue samples were immediately pulverized by hand with sterile, RNAse Away treated pestels, resuspended in Trizol (Sigma) and frozen at -80^{o} C. mRNA was extracted from NPI xenograft-bearing kidneys using Trizol reagent following the manufacturer's protocol (Invitrogen, Burlington, Ontario, Canada). cDNA was

constructed from 1 μg of total RNA using Superscript RNase H^- Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol and 1 μl of cDNA was amplified for 35 cycles using Taq DNA Polymerase (Invitrogen). The PCR conditions and the primer sequences were similar to what we have previously published (19). The PCR conditions were as follows: 30 seconds denaturation at 94^{o}C, 30 seconds annealing at 58^{o}C, 30 seconds extension at 72^{o}C and a final extension of 72^{o}C for 10 min followed by a 4^{o}C hold. Products were separated on an ethidium bromide stained 2% agarose gel and images captured with Alpha Digidoc software (Perkin-Elmer, Boston, USA). Primer sequences were outlined as follows: 5'- TGAGTGGCTGTCTTTTGACG-3' (forward) and 5'- TTGGTATCCAGGGCTCTCC-3' (reverse) (mTGFβ1-269 bp; accession no. NM_011577), 5'-CAAACAAAGGACCAGCTGGAC-3' (forward) and 5'-GAGTCCAGCAGACTCAATAC-3' (reverse) (IL10-406 bp, accession no. NM_010548), and 5'-AATCCCATCACCATCTTCCA-3' (forward) and 5'-GGCAGTGATGGCATGGACTG-3' (reverse) (GAPDH-310 bp, accession no. NM_008084). Positive control includes thymus cDNA from naïve B6 mice while the negative control includes kidney from these mice and water in place of experimental cDNA. GAPDH primers (housekeeping gene) ensured the integrity of cDNA and all the primer pairs spanned at least one intron to make sure that no genomic DNA was detected during the amplification. Products were separated on an ethidium bromide-stained 2% w/w agarose gel and images were captured with Alpha Digidoc software (Perkin-Elmer, Boston, MA, USA).

2.2.7 Detection of anti-porcine IgG antibodies by flow cytometry

The effect of combined mAb therapy on the humoral immune responses of B6 and reconstituted B6 *rag-/-* mice with NPI xenografts were determined by measuring the levels of mouse anti-porcine IgG antibodies in the serum samples of these mice using flow cytometry following our published method (14;18). Spleen cells ($1x10^6$) obtained from neonatal porcine islet donors were incubated with 1:128 dilutions of mouse serum for 1 h at 37°C, 5% CO_2, and 95% air. After incubation, spleen cells were then washed with PBS and incubated with 1:200 dilutions of FITC-conjugated rat adsorbed goat anti-mouse IgG antibody (Southern Biotechnology Associates, Inc. Birmingham, Alabama, AL, USA) for 1 h at 4°C. The percentage of cells bound to the antibody was detected from single parameter fluorescence histograms on a BD FACS Calibur flow cytometry machine (BD Biosciences Pharmingen) after gating on viable lymphocytes. Controls for this experiment include porcine spleen cells alone and spleen cells incubated with secondary antibody without mouse serum.

2.2.8 Characterization of lymphocytes by flow cytometry

Spleen from B6 and reconstituted B6 *rag-/-* transplanted with NPI were harvested on the day of rejection or at the end of the study. Spleen cells were isolated by dissociation of the tissue into single-cell suspension using mechanical disruption between rough edges of sterile glass slides. Red blood cells were depleted by incubation of spleen cells in red blood cell lyses buffer, absolute lymphocyte numbers were determined by staining the cells with Trypan blue exclusion dye, and live cells were counted using a hemocytometer (20). Aliquots

of 1 x 10^6 spleen cells were incubated for 30 min at 4˚C with fluorescent conjugated antibodies (1:100 dilutions, eBioscience, San Diego, CA, USA) specific for particular lymphocyte markers. Fluorescence histograms were created using a BD FACS Calibur flow cytometry machine (BD Biosciences Pharmingen, Mississauga, ON, Canada) and were used to determine the percentage of positive cells labeled with the corresponding antibodies. Controls for this experiment include spleen cells from tolerant or naïve B6 mice that were or were not incubated with any of the antibodies.

2.2.9 In vitro proliferation assays

The *in vitro* proliferation of T cells from naïve non-transplanted and NPI transplanted B6 mice were determined after stimulation with mitogen, anti-CD3ε antibody or donor pig spleen cells. Briefly, 5 x 10^5 B6 mouse recipient spleen cells were stimulated with Concanavalin A (ConA; 10 μg/ml, Sigma) or with mouse anti-CD3ε (10 μg/ml, eBioscience) or with irradiated (2,500 rads) donor neonatal pig spleen cells (3 x 10^5 cells). The cells were cultured in 96-well flat-bottom plates in a total volume of 0.2 ml of Eagle's modified essential medium (EMEM, Invitrogen, Burlington, ON, Canada) supplemented with 10% v/v FBS (Invitrogen), 1 x 10^{-5} mol/l 2-mercaptoethanol, 2 x 10^{-3} mol/l L-glutamine and 1% antibiotics in triplicate at 37°C, 5% CO_2 and 95% air. After 1-6 days (for ConA and anti-CD3ε mAb stimulation assays) and three, 4, and 5 days of culture for mixed lymphocyte reaction (MLR) assays, T cell proliferation was determined by pulsing the cells of primary culture with one μCi [^{3}H]-thymidine/well for 18 h. Cells were then harvested onto glass microfiber filters (Wallac, Turku, Finland),

and counts per minute (cpm) per sample were detected using the Wallac MicroBeta® TriLux luminescence counter (PerkinElmer, Waltham, MA, USA).

2.2.10 Adoptive transfer experiments

At 150 days post-transplantation, 50 x10^6 spleen cells from B6 mouse recipients of NPI that maintained long-term normoglycemia or from naïve non-transplanted B6 mice were injected into the peritoneum of some B6 *rag-/-* mouse recipients of NPI. Blood glucose levels of these mice were monitored three times a week for 60 days post-cell injection. At the time of rejection or at 60 days post-cell injection if mice remained normoglycemic, NPI xenografts were harvested and were examined for the presence of insulin positive cells as well as immune cells as described above. The blood glucose levels of those B6 *rag-/-* mice that maintained normoglycemia for 60 days post-cell injection were measured after removal of the kidney bearing the NPI xenograft. A return to the diabetic state was noted and the spleen cells of B6 *rag-/-* mouse recipients were isolated at the end of the study to confirm the presence of adoptively transferred immune cells using flow cytometry.

2.2.11 CFSE labeling of adoptively transferred spleen cells

Ten million per ml of spleen cells from B6 mouse recipients of NPI that maintained long-term normoglycemia or from naïve non-transplanted B6 mice were suspended in sterile PBS and incubated with 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR, USA) in the dark with periodic agitation at 37°C for 10 min. Excess CFSE was quenched with 5% v/v FBS (Sigma) and the cells were washed with PBS. Fifty million CFSE

labeled spleen cells in a total volume of 200 µl were injected into the peritoneum of B6 *rag-/-* mouse recipients of NPI or naïve non-transplanted B6 *rag-/-* mice. On the day of rejection or at 60 days post-cell injection, spleen cells were collected as described above and were incubated with PE-Cy5 conjugated anti-mouse TCR for two-color flow cytometric analysis. CFSE specific fluorescence histograms were created using a BD FACS Calibur flow cytometry machine (BD Biosciences Pharmingen) by gating on TCR^+ T cells.

2.2.12 Transplantation of a second party NPI

Some B6 mice that maintained normoglycemia for more than 100 days post-transplantation were re-transplanted with a second party NPI under the right kidney capsule. Blood glucose levels of these mice were monitored for another 100 days (200 days after the first NPI transplant) and at this time, the left kidney bearing the first NPI xenograft was removed. Blood glucose levels of these mice were measured for an additional of 100 days to monitor the function of the second party NPI xenograft. After 100 days (300 days after transplantation of the first NPI), the remaining right kidney that contains the second NPI xenograft was removed to determine that maintenance of long-term normoglycemia was due to the presence of the second party NPI xenograft.

2.2.13 Statistical analysis

Statistical differences between groups were sought using Mann-Whitney U test in SPSS statistical software, version 13.0 for Windows (Chicago, IL, USA). A p value of less than 0.05 was considered to be statistically significant.

2.3 RESULTS

2.3.1 Short-term administrations of a combination of anti-LFA-1 and anti-CD154 mAbs result in indefinite NPI xenograft survival in B6 mice.

To determine if short-term administrations of a combination of anti-LFA-1 and anti-CD154 mAbs can induce durable NPI xenograft protection we lengthen the metabolic follow-up period of B6 mice up to 300 days post-transplantation. All 50 NPI transplanted mice treated with the combination of mAbs achieved normoglycemia within 70 to 98 days post-transplantation and they maintained normoglycemia for 100 days post-transplantation (Table 2.1). At 150 days post-transplantation, which defines our standard endpoint of the study, 39 of 40 mice maintained normoglycemia and one mouse became diabetic at 105 days post-transplantation. At this time point, the ability of some recipients to respond to glucose challenge *in vivo* was performed (Figure 2.1A). The blood glucose levels of these recipients at the beginning of the challenge (time 0 min) and at the end of the challenge (time 90 and 120 min) were not significantly different with those observed in B6 *rag-/-* mouse recipients of the same NPI and naïve B6 mice. However, the blood glucose levels of both B6 and B6 *rag-/-* mouse recipients of the same NPI were significantly lower at 15, 30, and 60-min time points when compared with naïve B6 mice (n=5 in each group, $p<0.008$). At 200 days post-transplantation, 18 of 20 mice remained normoglycemic while two mice returned to the diabetic state at 160 and 185 days post-transplantation.

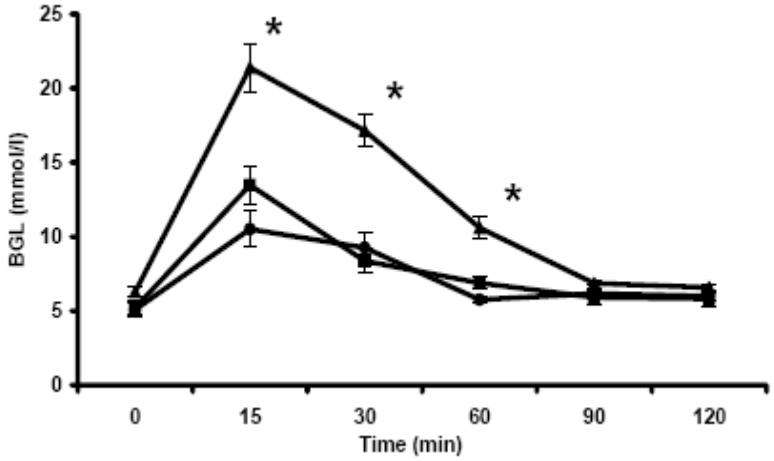

Figure 2.1: Blood glucose levels of B6 mouse recipients of NPI (black squares) responding to oral glucose challenge at 150 days post-transplantation. Control groups are age-matched B6 *rag-/-* mouse recipients of the same NPI (black circles) and naïve non-transplanted B6 mice (black triangles). n=5 in each group, * $p < 0.008$ vs. B6 and B6 *rag-/-* mouse recipients of NPI.

Nine out of 10 mice maintained normoglycemia at 250 days post-transplantation and one mouse became diabetic at 216 days post-transplantation. Finally, at 300 days post-transplantation, seven of eight recipients maintained normoglycemia and one recipient returned to hyperglycemic state at 266 days post-transplantation. Removal of the NPI xenograft-bearing kidney from randomly selected mouse recipients that maintained long-term normoglycemia at various time points post-transplantation resulted in return to the diabetic state confirming that maintenance of normoglycemia is dependent on the islet xenograft (Table 2.1).

NPI xenografts from B6 mice with long-term normoglycemia (Figure 2.2A) showed intact islets composed of abundant insulin positive cells comparable to those observed in B6 *rag-/-* mice transplanted with the same NPI (Figure 2.2B). In contrast, B6 mice that eventually became diabetic had massive

amount of mononuclear cell infiltrate with no intact islets remaining in the transplant site (Figure 2.2C)

Table 2.1: Long-term survival of NPI xenografts in B6 mice treated with short-term administration of a combination of anti-LFA-1 and anti-CD154 mAbs.

End point (days post-transplantation)	n	Graft survival (days post-transplantation)	% Graft survival
100	50	>100 (x50)	100
150	40	>150 (x39), 105	97.5
200	20	>200 (x18), 160, 185	90
250	10	>250 (x9), 216	90
300	8	>300 (x7), 266	85.7

Moreover, we observed few immune cells surrounding the islet xenografts in B6 mouse recipients and further characterization of these cells revealed that they express foxp3 (Figure 2.3A). However, we found little or no $foxp3^+$ positive cells in the NPI xenografts of these mice (Figure 2.3B). RT-PCR analysis of the NPI xenografts harvested from B6 mice with long-term graft function showed the expression of TGF-β1 and IL-10 transcripts (Figure 2.3C), suggesting that local production of these regulatory cytokines may be partly responsible for the protection induced by the combined mAb therapy.

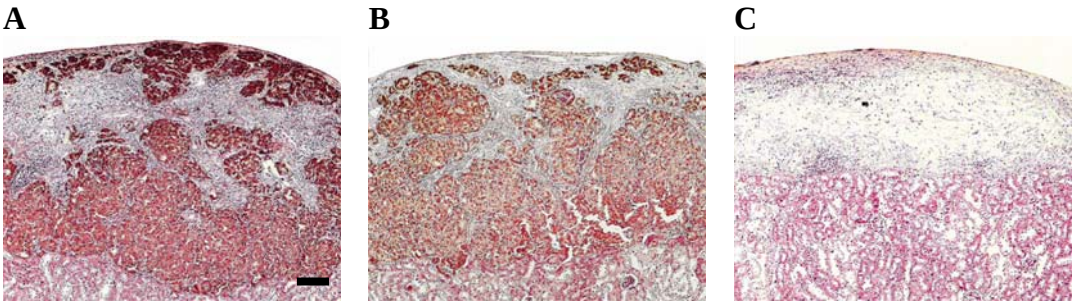

Figure 2.2: Insulin immunohistochemical staining of NPI xenografts in the B6 mice treated with short-term administration of a combination of anti-LFA-1 and anti-CD154 mAbs. Representative islet grafts from B6 (A) and B6 *rag-/-* (B) mouse recipients that had long-term normoglycemia contained intact islets with abundant insulin-positive cells (brown stain). Representative NPI xenografts from B6 mice that eventually rejected their grafts had no intact islets left at the graft site (C). Scale bar represents 100 μm.

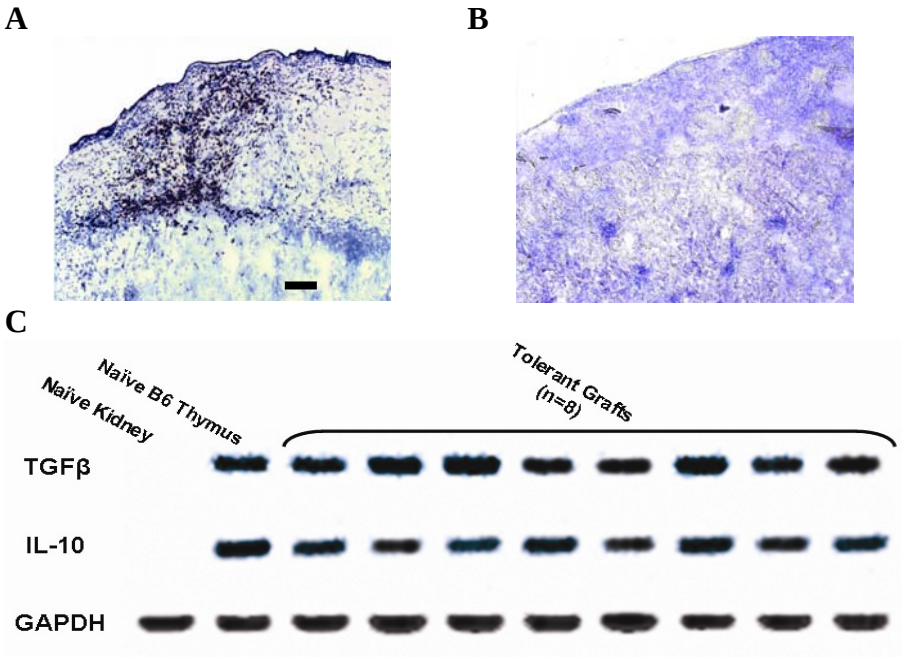

Figure 2.3: Immunohistological and RT-PCR characterization of the NPI xenografts. Immunohistological and RT-PCR characterization of the NPI xenografts from B6 mice with long-term graft function showed some foxp3^{+} cells (brown structures; A). However little or no foxp3^{+} cells in the islets grafts of the B6 mice which rejected the NPI xenografts eventually (B). Regulatory cytokine transcripts of TGFβ1 and IL-10 were detected on NPI xenografts from B6 mice with long-term normoglycemia at 150 days post-transplantation (C, n=8).

Positive control includes thymus cDNA from naïve non-transplanted B6 mice while the negative controls include kidney from naïve non-transplanted B6 mice and water in place of experimental cDNA. GAPDH serves as a housekeeping gene.

The levels of anti-porcine IgG antibodies in B6 mice that maintained long-term normoglycemia (Figure 2.4A) were comparable to the anti-porcine IgG antibody levels detected in naïve B6 mice (Figure 2.4B). In contrast, the anti-porcine IgG antibody levels of B6 mouse recipients that eventually rejected the NPI xenografts were significantly higher than those detected in B6 mice that maintained long-term normoglycemia (Figure 2.4C).

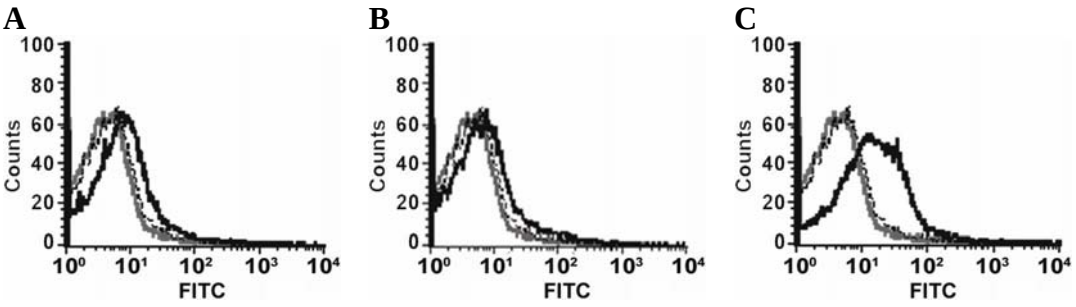

Figure 2.4: Mouse anti-porcine antibody levels in the treated B6 mouse recipients of NPI with mAbs. Representative histograms of the levels of anti-porcine IgG antibodies in B6 mice that maintained long-term normoglycemia (1.69%-5.07%, n=25, A) were comparable to those detected in naïve non-transplanted B6 mice (1.73%-4.57%, n=10, B) but were significantly ($p<0.001$) lower than those detected in B6 mice that eventually rejected the NPI (28.54%-43.69%, n=5, C). Controls for this experiment consisted of unstained spleen cells (dashed black line) and the spleen cells incubated just with secondary antibody without serum (solid gray line).

Taken together, these results indicate that short-term administrations of combined anti-LFA-1 and anti-CD154 mAbs induce tolerance to NPI xenografts.

2.3.2 Combined anti-LFA-1 and anti-CD154 mAb therapy results in changes in CD4$^+$ T cells expressing regulatory markers.

The total number of immune cells and proportions of CD4$^+$, CD8$^+$ T cells and CD19$^+$ B cells from spleen of tolerant B6 mice was comparable to those detected in naïve B6 mice (Table 2.2). Although the total number of immune cells and CD19$^+$ B cells in B6 mice that eventually rejected the graft was higher compared to those detected in tolerant B6 mice and naïve B6 mice, the difference was not statistically significant. In addition, while the frequencies of CD4$^+$ and CD8$^+$ T cells were lower in B6 mice that rejected the NPI xenografts compared to those observed in tolerant B6 and naive B6 mice, the difference was not statistically significant (Table 2.2).

Table 2.2: Phenotype of immune cells from B6 mouse recipients of NPI treated with a combination of anti-LFA-1 and anti-CD154 mAbs compared to the phenotype of the immune cells in naïve non-transplanted B6 mice and B6 mouse recipients of NPI that rejected their NPI xenografts.

Group	n	Mean ± SEM Total No. (x10^6)	Mean composition of isolated spleen cells (%) ± SEM		
			CD4$^+$	CD8$^+$	CD19$^+$
Naïve B6	12	89.61±2.15	20.98 ±0.82	13.04 ±0.40	55.18 ±2.0
Tolerant B6	19	86.02±2.34	20.21 ±0.66	13.32 ±0.47	56.65 ±2.07
Rejected B6	5	94.33±4.46	17.91 ±2.50	10.45 ±2.21	61.99 ±6.80

However, we found significant differences in the percentage of cells expressing CD4CD25 and CD8CD25 T cells between tolerant B6 mice, naïve B6 mice and B6 mice that eventually rejected the graft (Figure 2.5A). Further analysis of $CD4^+CD25^+$ T cells show a significant increase in the frequencies of these cells from tolerant B6 mice expressing foxp3 and GITR compared to those found in naïve B6 mice and B6 mice that eventually rejected the graft (Figure 2.5B). Similarly, the frequencies of $CD4^+$ T cells expressing PD-1 and CTLA-4 but not BTLA co-inhibitory markers were also significantly increased in tolerant B6 mice compared to those detected in the other groups (Figure 2.5C). However no significant difference in the frequencies of total $PD\text{-}1^+$, $CTLA\text{-}4^+$ and $BTLA^+$ spleen cells were found between tolerant B6 and naïve B6 mice (Figure 2.5D). A significant decrease of PD-1 and CTLA-4 but not BTLA expressions was detected in the spleen cells from B6 mice that eventually rejected the graft compared to those cells from tolerant and naïve B6 mice (Figure 2.5D). Collectively, these results indicate that combined anti-LFA-1 and anti-CD154 mAbs result in higher percentage of $CD4^+$ T cells expressing regulatory markers suggesting that tolerance to NPI xenografts may be mediated by T regulatory cells.

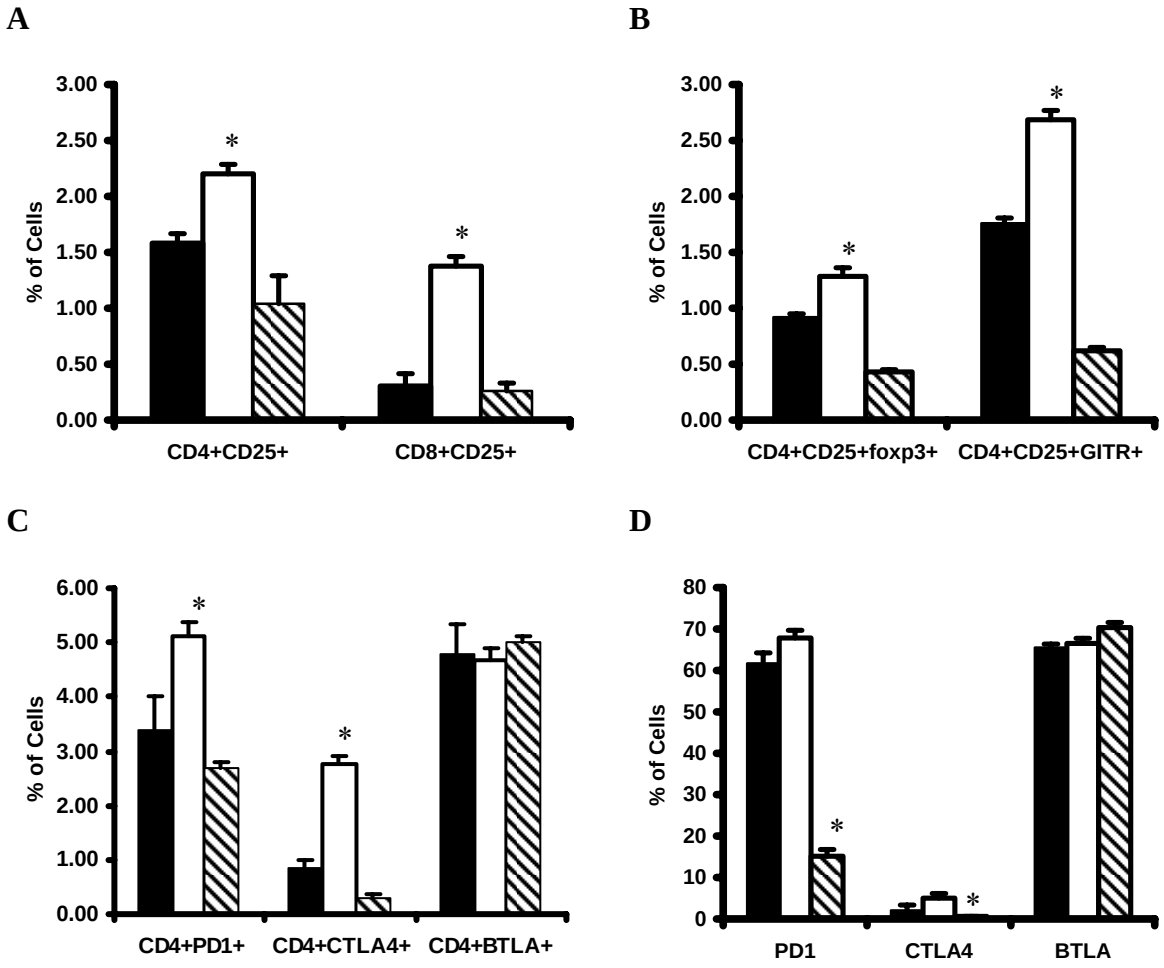

Figure 2.5: High frequency of $CD4^+$ T cells expressing regulatory markers were detected in B6 mice with long-term graft function. The frequency of $CD4^+CD25^+$ (2.20±0.08%, n=19) and $CD8^+CD25^+$ (1.38±0.09%, n=19) T cells in B6 mice with long-term graft function (white bars) was significantly ($p<0.0001$) higher than those observed in naïve non-transplanted B6 mice (black bars, 1.58±0.08% and 0.30±0.11%, respectively, n=12) and B6 mice that eventually rejected the graft (white downward diagonal bars, 1.04±0.25% and 0.26±0.07%, respectively, n=5, A). In this population there were also significantly more cells expressing foxp3 (1.29±0.08%, n=19, $p=0.0013$) and GITR (2.69±0.08%, n=19, $p<0.0001$) compared to those found in naïve non-transplanted B6 mice (0.92±0.03% and 1.76±0.05%, respectively, n=12) and B6 mice that eventually rejected the graft (white downward diagonal bars, 0.43±0.02% and 0.62±0.03%, respectively, n=5, B). The frequencies of $CD4^+$ T cells expressing PD-1 (5.12±0.24%, n=19) and CTLA-4 (2.78±0.12%, n=19) but not BTLA (4.69±0.23%, n=19) co-inhibitory markers were also significantly ($p<0.0001$) increased in B6 mice with long-term NPI xenograft survival compared to those detected in naïve non-transplanted B6 mice (3.38±0.63%, 0.83±0.18%, 4.79±0.55%, n=12, respectively) and B6 mice that eventually rejected the graft

(white downward diagonal bars, 2.68±0.13%, 0.31±0.06%, 5.01±0.11%, respectively, n=5, C). Significantly less ($p<0.0001$) cells expressing PD-1 (15.15±1.60%, n=5) and CTLA-4 (0.61±0.01%, n=5) but not BTLA (70.36±1.22%, n=5) were detected in B6 mice that eventually rejected the graft (white downward diagonal bars) compared to those found in naïve non-transplanted B6 mice (black bars,61.68±2.55%, 2.04±0.61% and 65.50±0.88%, respectively, n=12) and tolerant B6 mice (white bars, 67.82±0.79%, 5.01±1.13%, and 66.53±1.23%, respectively, n=19, D).

2.3.3 Combined anti-LFA-1 and anti-CD154 mAb therapy result in T regulatory cell-mediated tolerance to NPI xenografts.

To better define the role of T regulatory cells in protection generated by combined anti-LFA-1 and anti-CD154 mAbs, a group of tolerant B6 mice was treated with depleting anti-CD25 mAb beginning at 150 days post-transplantation. All normoglycemic recipients became diabetic at 25.3 ± 2.5 days post-injection of anti-CD25 mAb (n=7, Figure 2.6A). NPI xenografts from these mice had infiltrating immune cells no insulin-positive cells remaining in the grafts (Figure 2.6B). The levels of mouse anti-porcine IgG antibody in these mice were significantly higher (Figure 2.6C) than those detected in tolerant B6 mice without anti-CD25 mAb treatment and naïve B6 mice (Figure 2.4A and B, respectively).

These results further support that T regulatory cells mediate long-term immune protection of NPI xenografts rendered by combined anti-LFA-1 and anti-CD154 mAb therapy.

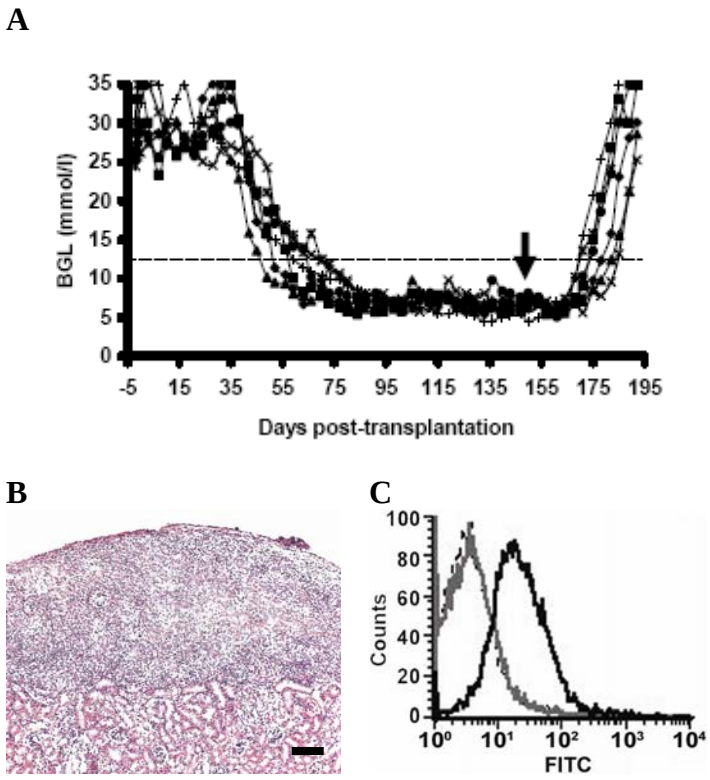

Figure 2.6: T regulatory cells mediate long-term immune protection of NPI xenografts rendered by combined anti-LFA-1 and anti-CD154 mAb therapy. All normoglycemic recipients (n=7) that received depleting anti-CD25 mAb on 0, 2, 4 and 6 days post-administration beginning at 150 days post-transplantation (arrow) became diabetic at 175.3 ± 2.5 days post-transplantation (25.3 ± 2.5 days post-injection of anti-CD25 mAb; A). NPI xenografts had infiltrating immune cells and absence of insulin-positive cells (B). Scale bar represents 100 μm. The levels of mouse anti-porcine IgG antibody in these mice were significantly ($p<0.0001$) higher (37.28±1.80%, n=7, C) compared to the levels detected in tolerant B6 mice not treated with anti-CD25 mAb and naïve non-transplanted B6 mice (Figure 1G and H, respectively). Representative histograms are shown and controls for this experiment consisted of unstained spleen cells (dashed black line) and spleen cells incubated just with secondary antibody without serum (solid gray line).

2.3.4 Lymphocytes from tolerant mice suppress the in vitro proliferation of pig-primed lymphocytes in a dose-dependent manner.

Lymphocytes from spleen of tolerant B6 mice responded robustly after non-antigen specific stimulation with Con A and anti-CD3ε mAb (Figure 2.7A). The responses are comparable to the proliferative responses of lymphocytes from spleen of naïve B6 mice after addition of the same stimulator molecules (Figure 2.7B). Similar to what we have previously reported (21), we observed no measurable proliferation of lymphocytes from naïve B6 mice several days after stimulation with pig spleen cells (Figure 2.7C). We also found that lymphocytes from spleen of tolerant B6 mice did not proliferate after stimulation with pig spleen cells from the islet donor (Figure 2.7C). However, we found that initial priming of naïve B6 mice with pig spleen cells resulted in strong proliferation of lymphocytes from these mice when re-stimulated with pig spleen cells *in vitro* (Figure 2.7C).

To determine whether lymphocytes from tolerant mice are capable of suppressing the proliferation of lymphocytes from pig-primed B6 mice, we mixed different ratios of the two cell populations.

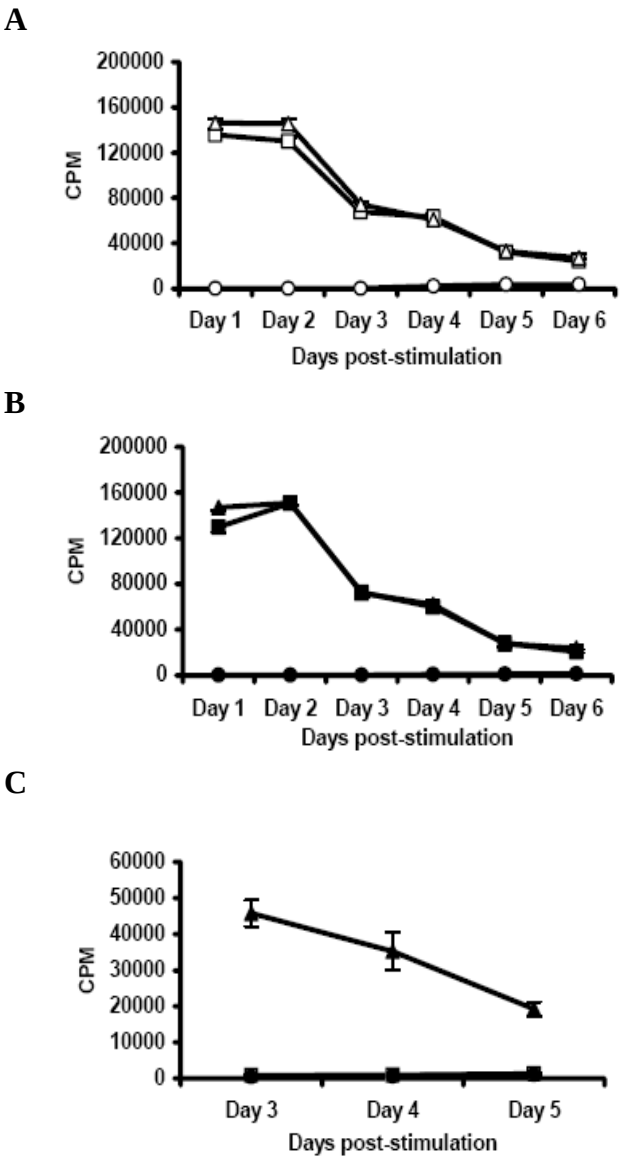

Figure 2.7: Lymphocytes from tolerant mice are unresponsiveness after stimulation with pig spleen cells *in vitro*. Lymphocytes from tolerant B6 mice (n=9, A) responded robustly after stimulation with ConA (white squares) or anti-CD3ε mAb (white triangles) comparable to what was observed in lymphocytes from naïve non-transplanted B6 mice (black squares for ConA and black triangles for anti-CD3ε mAb, n=9, B). Lack of proliferation was observed when these cells were not stimulated (white circles for lymphocytes from tolerant mice (A) and black circles for naïve mice (B)). Lymphocytes from pig-primed B6 mice (n=6) responded robustly when stimulated with pig spleen cells (black triangles, C). While lymphocytes from tolerant B6 mice (white squares, n=9) and naïve non-transplanted B6 mice (black circles, n=9) did not proliferate after stimulation with pig spleen cells from the islet donor (C).

Addition of lymphocytes from tolerant B6 mice resulted in a dose-dependent inhibition of proliferation of lymphocytes from pig-primed B6 mice (Figure 2.8A). At 1:1, 1:2, 1:4, and 1:8, but not 1:16 ratios of lymphocytes from tolerant B6 mice to lymphocytes from pig-primed B6 mice, we observed significant inhibition of proliferation of the latter cell population at 3 days post-culture. In contrast, addition of lymphocytes from naïve B6 mice to lymphocytes from pig-primed B6 mice at similar ratios did not result in the inhibition of proliferation but resulted in proliferation of lymphocytes from pig-primed B6 mice (Figure 2.8B).

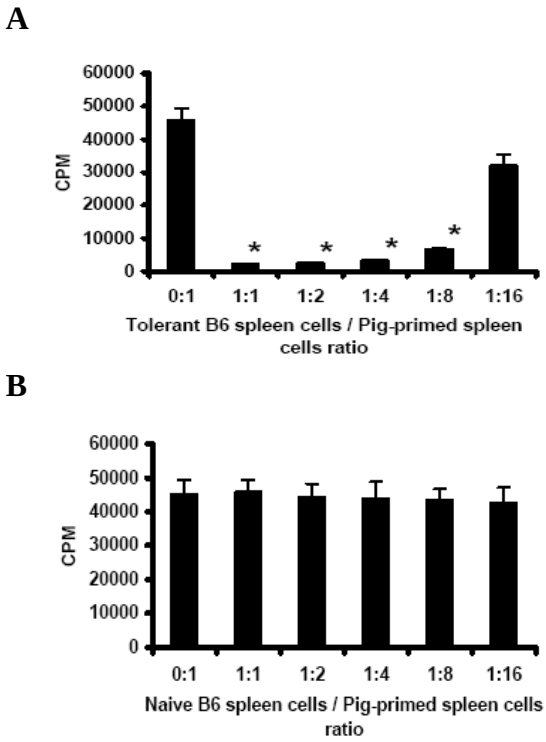

Figure 2.8: Lymphocytes from tolerant mice suppress the *in vitro* proliferation of pig-primed lymphocytes in a dose-dependent manner. Lymphocytes from tolerant B6 mice significantly (*$p<0.0005$, $n=3$) inhibited the proliferation of pig-primed lymphocytes in a dose-dependent manner on day three post-culture (A). However addition of lymphocytes from naïve B6 mice to lymphocytes from pig-primed B6 mice at similar ratios did not result in the inhibition of proliferation but resulted in proliferation of lymphocytes from pig-primed B6 mice (B).

2.3.5 Tolerance rendered by combined anti-LFA-1 and anti-CD154 mAb therapy can be extended to second party NPI xenografts.

All B6 *rag-/-* mice transplanted with first and second party NPI remained normoglycemic for more than 60 days after transfer of spleen cells from tolerant B6 mice (Table 2.3). However, B6 *rag-/-* mice with established NPI xenografts reconstituted with spleen cells from naïve B6 mice rejected their grafts by 15 days post-cell transfer (Table 2.3).

Table 2.3: Transfer of tolerance induced by a combination of anti-LFA-1 and anti-CD154 mAbs to NPI xenografts.

Source of islets	Source of spleen cells	n	Graft survival (days post- cell transfer)
First or second party neonatal pig	Naïve non-transplanted B6 mice	8	10, 11(x2), 13(x2), 15(x3)
First party neonatal pig	B6 mouse recipients with long-term normoglycemia	9	>60(x9)
Second party neonatal pig	B6 mouse recipients with long-term normoglycemia	10	>60(x10)

CFSE labeling of T cells from spleen of these mice, revealed that they exhibit robust proliferation (Figure 2.9A). In contrast, CFSE labeling of T cells from B6 mice tolerant to first party NPI xenografts showed that a large proportion of these cells did not proliferate robustly after injection into B6 *rag-/-* mice with established first (Figure 2.9B) and second (Figure 2.9C) party NPI xenografts.

However, in the absence of NPI xenografts, these cells were able to proliferate when injected into naïve B6 *rag-/-* mice (Figure 2.9D).

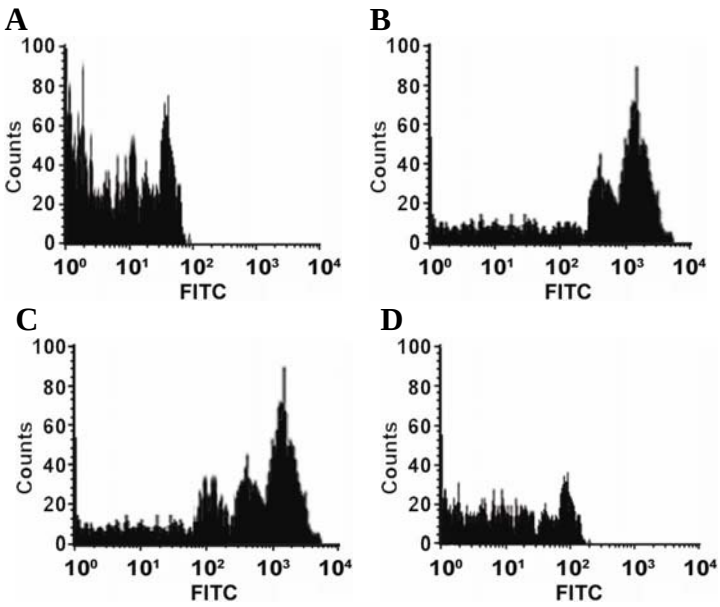

Figure 2.9: Lymphocytes from tolerant mice show a limited proliferation *in vivo*. CFSE labeling of T cells from spleen of naïve B6 mice that rejected the NPI xenografts, showed robust T cell proliferation (A). In contrast, CFSE labeling of T cells from B6 mice tolerant to first party NPI xenografts showed that a large proportion of these cells did not proliferate robustly after injection into B6 *rag-/-* mice with established first (B) and second (C) party NPI xenografts. However, in the absence of NPI xenografts, these cells were able to proliferate when injected into naïve B6 *rag-/-* mice (D).

The first (Figure 2.10A) and second (Figure 2.10B) party NPI xenografts from B6 *rag-/-* mice reconstituted with spleen cells from tolerant B6 mice had intact islets composed of abundant insulin positive cells and immune cells surrounding but not infiltrating the NPI xenografts. However, massive immune cellular infiltrate and no insulin positive cells were detected in the NPI grafts from B6 *rag-/-* mouse recipients injected with spleen cells from naïve B6 mice (Figure 2.10C).

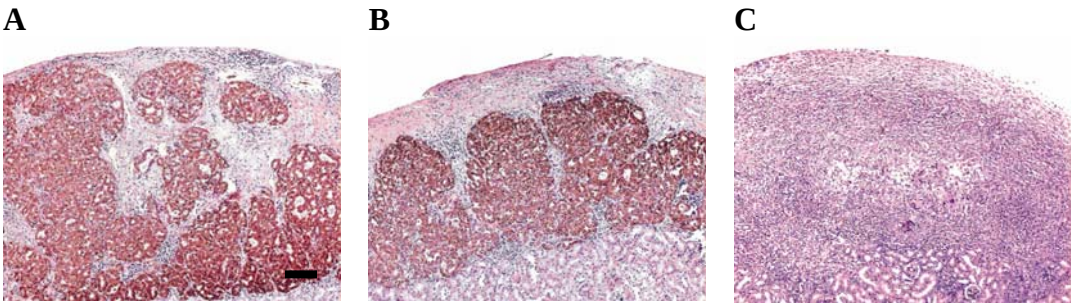

Figure 2.10: Insulin staining of the NPI xenografts from B6 *rag-/-* mice injected with lymphocytes from tolerant B6 mice. Representative first (A) and second (B) party NPI xenografts from B6 *rag-/-* mice injected with lymphocytes from tolerant B6 mice had intact islets composed of abundant insulin positive cells which were absent in B6 *rag-/-* mouse recipients reconstituted with spleen cells from naïve non-transplanted B6 mice (C). Scale bar represents 100 μm.

The humoral immune responses in B6 *rag-/-* mice with first (Figure 2.11A) and second (Figure 2.11B) party NPI xenografts that maintained normoglycemia for more than 60 days after injection of spleen cells from tolerant B6 mice were comparable. The IgG antibody levels of these mice however, were significantly lower than those detected in B6 *rag-/-* mice that rejected the NPI xenografts after injection of spleen cells from naïve B6 mice (Figure 2.11C).

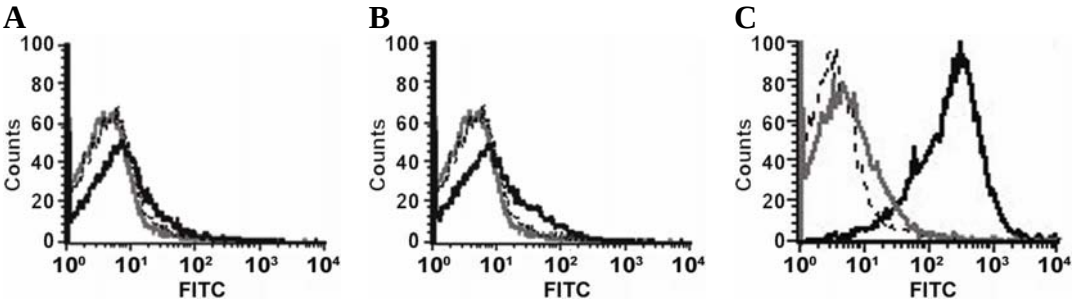

Figure 2.11: The humoral responses of lymphocytes from tolerant B6 mice injected into B6 *rag-/-* mouse recipients of NPI xenografts. The humoral immune responses of B6 *rag-/-* mouse recipients of first (A, n=9) and second party (B, n=10) NPI xenografts 60 days after reconstitution with lymphocytes from tolerant B6 mice were comparable (6.31±0.59% and 7.55±1.02%, respectively). The IgG antibody levels from these mice were significantly

($p<0.0001$) lower than the levels of anti-porcine IgG antibody levels of B6 *rag-/-* mice (67.42±5.38%, n=8) that rejected the NPI xenografts by 15 days post injection of lymphocytes from naïve non-transplanted B6 mice (C). Controls for this experiment consisted of unstained spleen cells (dashed black line) and spleen cells incubated just with secondary antibody without serum (solid gray line).

To confirm that tolerance to first party NPI xenografts can be extended to the second party NPI xenografts, tolerant B6 mice were re-transplanted with a second party NPI. All of the mice transplanted with a second party NPI xenograft maintained normoglycemia for more than 100 days post-harvest of the first party NPI xenograft. However, removal of the right kidney bearing the second party NPI xenograft resulted in the recurrence of diabetes in all B6 mouse recipients (Figure 2.12A) indicating that tolerance induced by combined anti-LFA-1 and anti-CD154 mAbs to first party NPI xenografts can be extended to second party NPI xenografts. The first (Figure 2.12B) and second (Figure 2.12C) party NPI xenografts from tolerant B6 mice had intact islets composed of abundant insulin positive cells. Low levels of anti-porcine IgG antibodies (Figure 2.12D) were detected in these mice at the end of the study (>300 and >200 days post-transplantation of the first and second party NPI, respectively).

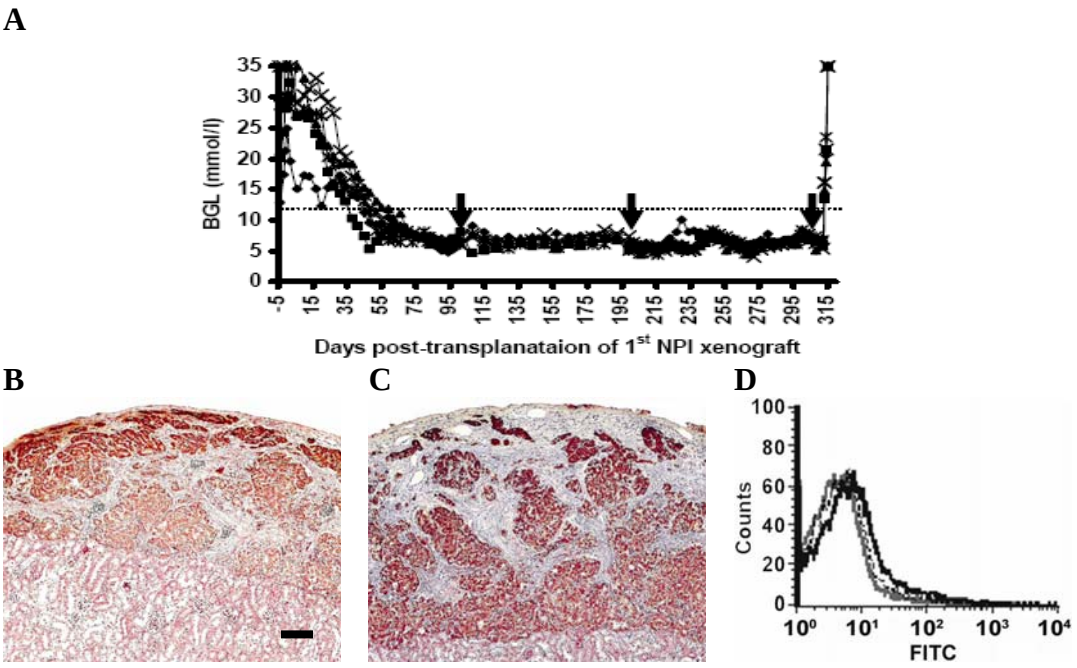

Figure 2.12: Tolerance provided by combined anti-LFA-1 and anti-CD154 mAb therapy can be extended to second party NPI xenografts. Tolerant B6 mice (n=5) maintained normoglycemia after re-transplantation of a second party NPI xenograft (first arrow, A) and remained normoglycemic after removal of the first party NPI xenograft (second arrow). These mice became diabetic only after removal of the right kidney bearing the second party NPI xenograft (third arrow). Representative first (B) and second (C) party NPI xenografts from tolerant B6 mice had intact islets composed of abundant insulin positive cells. Scale bar represents 100 µm. Low levels of anti-porcine IgG antibodies (3.95±0.52%, n=5) were detected in these mice at the end of the study (>300 and >200 days post-transplantation of the first and second party NPI, respectively, D). Representative histograms for porcine cells bound to mouse IgG antibodies are shown and controls for this experiment consisted of unstained porcine spleen cells (dashed black line) and the cells incubated just with secondary antibody without without serum (solid gray line).

2.4 DISCUSSION

One of the major challenges in making xenotransplantation of NPI a clinical reality is overcoming the rejection barrier using safe anti-rejection regimens and eventually, tolerance induction strategies to minimize if not alleviate the continuous use of harmful immunosuppressive drugs. Our study showed for the first time that targeting the adhesion and costimulatory pathways of T cell activation using short-term administrations of anti-LFA-1 and anti-CD154 mAbs could induce robust tolerance to NPI xenografts in B6 mice. Tolerant B6 mice could respond well to glucose challenge and a significant decrease in the blood glucose levels of these mice as well as in B6 *rag-/-* mouse recipients was observed at early time points after glucose challenge compared to what was observed in naïve B6 mice. This observation was perhaps due to the excess in β cell mass in the transplanted animals compared to the β cell mass that existed in the native pancreas of age-matched naïve B6 mice (13).

Long-term normoglycemia in tolerant B6 mice was associated with intact NPI xenografts containing numerous insulin-positive cells and mononuclear cells surrounding but not infiltrating the islet grafts. These NPI xenografts contained foxp3$^+$ cells as well as IL-10 and TGF β cytokine transcripts indicating the presence of T regulatory cells in the graft site (22-24). In addition, tolerance to NPI xenografts is associated with lower levels of xenoreactive anti-porcine IgG antibodies comparable to those detected in naïve B6 mice. We also found no significant difference in the total number of spleen cells and percentage of CD4$^+$, CD8$^+$ T cells as well as B cells from tolerant B6 mice compared to naïve B6 mice

suggesting that tolerance due to clonal deletion is unlikely. However, further analysis of the phenotype of immune cells from tolerant B6 mice using flow cytometry showed higher percentage of $CD4^+$ T cells, co-expressing cell surface markers for T regulatory cells (22;25-28) compared to those observed in naïve B6 mice. The importance of T regulatory cells in tolerance to NPI xenografts induced by the combined mAb therapy was further confirmed when tolerant B6 mice became diabetic after depletion of $CD25^+$ cells using mAb. All tolerant B6 mice became diabetic and their NPI xenografts had no insulin-positive cells but contained immune cells infiltrating the islet grafts.

We also demonstrate that by immunization of naïve non-transplanted B6 mice with pig spleen cells for 30 days, mouse T cells could proliferate strongly after stimulation with the same pig cells *in vitro*. We showed that T cells from tolerant B6 mice did not proliferate after stimulation with pig spleen cells. However, they were able of proliferating robustly after stimulation with non-antigen-specific stimulation with Con A or anti-CD3ε mAb indicating that the *in vitro* unresponsiveness observed in T cells from tolerant B6 mice is specific to pig antigens. Furthermore, cells from tolerant B6 mice were able to suppress the proliferative response of lymphocytes from B6 mice immunized with the same pig spleen cells that were used as stimulator cells in the MLR assays. These results indicate that unresponsiveness of T cells from tolerant B6 mice is not due to their anergic condition but could be due their regulatory function (29;30). Our results also showed for the first time that tolerance to first party NPI xenografts could be extended to second party NPI xenografts as demonstrated by transfer of

protection to NPI xenografts and survival of a second party NPI xenografts in tolerant B6 mice. CFSE labeling of lymphocytes showed limited *in vivo* proliferation of a large proportion of T cells from tolerant B6 mice when transferred into B6 *rag-/-* mice with established first or second party NPI xenografts. Taken together, our study demonstrates for the first time that targeting the adhesion and costimulatory pathways for T cell activation by short-term administrations of anti-LFA-1 and anti-CD154 mAbs can induce tolerance to NPI xenografts in immune competent B6 mice through T regulatory cells. Combination of biologic agents that interferes with these pathways may be a promising strategy for induction of tolerance to islet xenografts and could form a significant component of future anti-rejection regimens in clinical islet xenotransplantation.

2.5 REFRERENCES

1. Shapiro,AM, Lakey,JR, Ryan,EA, Korbutt,GS, Toth,E, Warnock,GL, Kneteman,NM, Rajotte,RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N.Engl.J.Med.* 343:230-238, 2000

2. Shapiro,AM, Ricordi,C, Hering,BJ, Auchincloss,H, Lindblad,R, Robertson,RP, Secchi,A, Brendel,MD, Berney,T, Brennan,DC, Cagliero,E, Alejandro,R, Ryan,EA, DiMercurio,B, Morel,P, Polonsky,KS, Reems,JA, Bretzel,RG, Bertuzzi,F, Froud,T, Kandaswamy,R, Sutherland,DE, Eisenbarth,G, Segal,M, Preiksaitis,J, Korbutt,GS, Barton,FB, Viviano,L, Seyfert-Margolis,V, Bluestone,J, Lakey,JR: International trial of the Edmonton protocol for islet transplantation. *N.Engl.J.Med.* 355:1318-1330, 2006

3. Ryan,EA, Paty,BW, Senior,PA, Bigam,D, Alfadhli,E, Kneteman,NM, Lakey,JR, Shapiro,AM: Five-year follow-up after clinical islet transplantation. *Diabetes* 54:2060-2069, 2005

4. Molinari,M, Al Saif,F, Ryan,EA, Lakey,JR, Senior,PA, Paty,BW, Bigam,DL, Kneteman,NM, Shapiro,AM: Sirolimus-induced ulceration of the small bowel in islet transplant recipients: report of two cases. *Am.J.Transplant.* 5:2799-2804, 2005

5. Senior,PA, Paty,BW, Cockfield,SM, Ryan,EA, Shapiro,AM: Proteinuria developing after clinical islet transplantation resolves with sirolimus withdrawal and increased tacrolimus dosing. *Am.J.Transplant.* 5:2318-2323, 2005

6. Ryan,EA, Paty,BW, Senior,PA, Shapiro,AM: Risks and side effects of islet transplantation. *Curr.Diab.Rep.* 4:304-309, 2004

7. Alfadhli,E, Koh,A, Albaker,W, Bhargava,R, Ackerman,T, McDonald,C, Ryan,EA, Shapiro,AM, Senior,PA: High prevalence of ovarian cysts in premenopausal women receiving sirolimus and tacrolimus after clinical islet transplantation. *Transpl.Int.* 2009

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

9. Froud,T, Ricordi,C, Baidal,DA, Hafiz,MM, Ponte,G, Cure,P, Pileggi,A, Poggioli,R, Ichii,H, Khan,A, Ferreira,JV, Pugliese,A, Esquenazi,VV, Kenyon,NS, Alejandro,R: Islet transplantation in type 1 diabetes mellitus using cultured islets and steroid-free immunosuppression: Miami experience. *Am.J.Transplant.* 5:2037-2046, 2005

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

11. Pileggi,A, Ricordi,C, Kenyon,NS, Froud,T, Baidal,DA, Kahn,A, Selvaggi,G, Alejandro,R: Twenty years of clinical islet transplantation at the Diabetes Research Institute--University of Miami. *Clin.Transpl.*177-204, 2004

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

13. Korbutt,GS, Elliott,JF, Ao,Z, Smith,DK, Warnock,GL, Rajotte,RV: Large scale isolation, growth, and function of porcine neonatal islet cells. *J.Clin.Invest* 97:2119-2129, 1996

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

15. Kobayashi,T, Harb,G, Rayat,GR: 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, 2005

16. Kin,T, Korbutt,GS, Kobayashi,T, Dufour,JM, Rajotte,RV: Reversal of diabetes in pancreatectomized pigs after transplantation of neonatal porcine islets. *Diabetes* 54:1032-1039, 2005

17. Cardona,K, Korbutt,GS, Milas,Z, Lyon,J, Cano,J, Jiang,W, Bello-Laborn,H, Hacquoil,B, Strobert,E, Gangappa,S, Weber,CJ, Pearson,TC, Rajotte,RV, Larsen,CP: Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat.Med.* 12:304-306, 2006

18. Arefanian,H, Tredget,EB, Rajotte,RV, Korbutt,GS, Gill,RG, Rayat,GR: Combination of anti-CD4 with anti-LFA-1 and anti-CD154 monoclonal antibodies promotes long-term survival and function of neonatal porcine islet xenografts in spontaneously diabetic NOD mice. *Cell Transplant.* 16:787-798, 2007

19. Kobayashi,T, Harb,G, Rajotte,RV, Korbutt,GS, Mallett,AG, Arefanian,H, Mok,D, Rayat,GR: Immune mechanisms associated with the rejection of encapsulated neonatal porcine islet xenografts. *Xenotransplantation* 13:547-559, 2006

20. Tredget,EB, Arefanian,H, Gill,RG, Rajotte,RV, Rayat,GR: Monotherapy with anti-LFA-1 monoclonal antibody promotes long-term survival of rat islet xenografts. *Cell Transplant.* 17:599-608, 2008

21. Rayat,GR, Johnson,ZA, Beilke,JN, Korbutt,GS, Rajotte,RV, Gill,RG: The degree of phylogenetic disparity of islet grafts dictates the reliance on indirect CD4 T-cell antigen recognition for rejection. *Diabetes* 52:1433-1440, 2003

22. Hori,S, Nomura,T, Sakaguchi,S: Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061, 2003

23. Dieckmann,D, Bruett,CH, Ploettner,H, Lutz,MB, Schuler,G: Human CD4(+)CD25(+) regulatory, contact-dependent T cells induce interleukin 10-producing, contact-independent type 1-like regulatory T cells [corrected]. *J.Exp.Med.* 196:247-253, 2002

24. Nakamura,K, Kitani,A, Strober,W: Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J.Exp.Med.* 194:629-644, 2001

25. Sakaguchi,S, Sakaguchi,N, Asano,M, Itoh,M, Toda,M: Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J.Immunol.* 155:1151-1164, 1995

26. McHugh,RS, Whitters,MJ, Piccirillo,CA, Young,DA, Shevach,EM, Collins,M, Byrne,MC: CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity.* 16:311-323, 2002

27. Keir,ME, Butte,MJ, Freeman,GJ, Sharpe,AH: PD-1 and its ligands in tolerance and immunity. *Annu.Rev.Immunol.* 26:677-704, 2008

28. Read,S, Malmstrom,V, Powrie,F: Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J.Exp.Med.* 192:295-302, 2000

29. Quill,H: Anergy as a mechanism of peripheral T cell tolerance. *J.Immunol.* 156:1325-1327, 1996

30. Qin,S, Cobbold,SP, Pope,H, Elliott,J, Kioussis,D, Davies,J, Waldmann,H: "Infectious" transplantation tolerance. *Science* 259:974-977, 1993

CHAPTER 3

SPECIES AND TISSUE-SPECIFIC TOLERANCE TO NEONATAL PORCINE ISLET XENOGRAFTS INDUCED BY A COMBINATION OF ANTI-LFA-1 AND ANTI-CD154 MONOCLONAL ANTIBODIES IN C57BL/6J MICE

A version of this chapter has been submitted for publication. Arefanian, H, et al. *Diabetes* 10.2337/DB09-0898: 2009

3.1 INTRODUCTION

The widespread application of islet transplantation as treatment for type 1 diabetes is currently limited by the shortage of human donor islets and the chronic use of harmful immunosuppressive drugs to prevent rejection (1,2). Neonatal porcine islets (NPI) are being considered as an alternative source of islets for clinical transplantation because abundant number of islets can be easily isolated and maintained in culture (3). In addition they are capable of reversing diabetes in both small (3-5) and large animals (6,7), including the pre-clinical non-human primate model (7). We previously demonstrated that combined anti-LFA-1 and anti-CD154 monoclonal antibody (mAb) therapy resulted in a robust form of NPI xenograft protection in B6 mice (4,5,8). Recently, we also demonstrated in these mice that short-term administrations of combined anti-LFA-1 and anti-CD154 mAbs induced tolerance to NPI xenografts that is mediated by T regulatory cells (8). Tolerance to porcine islet xenografts is important to achieve so that the need for continuous use of harmful immunosuppressive drugs could be minimized or eliminated. In addition, it is important to assess if tolerance induced could be extended to a second party porcine islet xenografts in case human islet transplant recipients would require a second islet transplant to maintain insulin independence. It is also important to determine whether tolerance induced could be extended to different tissue or organ grafts without any additional anti-rejection therapy since type 1 diabetic patients are susceptible to developing deleterious secondary tissue or organ complications that may require replacement by transplantation. Thus, the aim of this study was to determine the species and

tissue specificity of tolerance induced by transient perturbation of adhesion and co-stimulatory pathways using short-term administrations of a combination of anti-LFA-1 and anti-CD154 mAbs. Our results show that tolerance induced by this strategy is specific to neonatal porcine islet but not to porcine skin xenografts.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Six to 8-week-old male immune-competent B6 (C57BL/6J, H-2^b) and immune-deficient B6 *rag-/-* (B6.129S7-$Rag1^{tm1Mom}$/J, H-2^b) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and were used as recipients. These mice were rendered diabetic by a single intraperitoneal injection of 180 or 175 mg/kg body weight of streptozotocin (STZ, Sigma, St Louis, MO, USA) for B6 or B6 *rag-/-* mice, respectively and monitored following our published protocol (8). All mice were fed standard laboratory food and cared for according to the guidelines established by the Canadian Council on Animal Care Committee. Islet or skin donors include 3-day-old Duroc cross neonatal porcine (>1.5 kg body weight), 8-10-week-old male BALB/c (BALB/$c^{Cr//AltBM}$, H-2^d) both were purchased from the University of Alberta (Edmonton, Alberta, Canada), and male Wistar-Furth rats (WF, $RT1^U$, 200-300 g body weight) that were purchased from Harlan Laboratories (Indianapolis, IN, USA).

3.2.2 Islet isolation and transplantation

Islets were isolated as previously described (3,9-12). Briefly, rodent islets were isolated via distension of the pancreas, followed by collagenase (Sigma, Oakville, ON) digestion, density gradient centrifugation with ficoll (Sigma,

Oakville, ON) and handpicking of the islets. Neonatal pigs were anesthetized with halothane and subjected to laparotomy and exsanguination. The pancreas was removed, placed in Hanks' balanced salt solution (HBSS, Sigma, St Louis, MO, USA) cut into small pieces, and digested with 2.5 mg/ml collagenase (clostridiopeptidase A, type XI, Sigma-Aldrich, St. Louis, MO, USA). Digested tissue was filtered through a 500 μm nylon screen then cultured for 7 days in HAM's F10 medium (GIBCO Laboratories, Grand Island, NY, USA) containing 10 mmol/l glucose, 50 μmol/l isobutylmethylxanthine (ICN Biomedicals, Montreal, Canada), 0.5% bovine serum albumin (fraction V, radioimmunoassay grade; Sigma), 2 mmol/l L-glutamine, 3 mmol/l $CaCl_2$, 10 mmol/l nicotinamide (BDH Biochemical, Poole, England), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C (5% CO_2, 95% air). A total of 500 freshly isolated rodent islets or 7 days cultured 2,000 NPI were transplanted under the left kidney capsule of diabetic B6 or B6 *rag-/-* mice as described previously (3,8,9,13). Briefly, diabetic B6 or B6 *rag-/-* mice were anesthetized by inhalational isoflurane and the left flank was shaved and sterilized with 100% ethanol. A flank incision was made lateral to the left paraspinal muscles and the peritoneum was sharply divided to expose the left kidney. A sterile cotton swab was utilized to expose and produce the left kidney from the incision. A small incision was made in the kidney capsule with a 27-gauge needle and a subcapsular pocket was expanded with the use of a sterile Pasteur pipette. Aliquots of 500 rodent islets or 2,000 NPI were aspirated into polyethylene (PE-50) tubing, pelleted by centrifugation and placed within the subcapsular pocket with the aid of a micromanipulator syringe.

Once the tubing was removed, the kidney capsule was cauterized with a disposable high-temperature cautery pen (Aaron Medical Industries, St. Petersburg, FL, USA). Engraftment was considered successful when blood glucose level reached ≤8.5 mmol/l. Graft rejection was defined as the first of three consecutive days of hyperglycemia (>12 mmol/l), and rejection was confirmed by histological analysis of the graft. Nephrectomy of the graft-bearing kidney was performed on recipients with long-term graft function to confirm that normoglycemia was due to the islet xenograft.

3.2.3 Monoclonal antibody therapies

B6 mouse recipients of NPI were randomly designated to receive short-term intra-peritoneal injections of anti-LFA-1 mAb (KBA; rat IgG2a) at 200 μg on days 0, 1, 7, 14 post-transplant plus anti-CD154 mAb (MR-1; hamster IgG1; Bio Express, West Lebanon, NH, USA) at 250 μg on days –1 and 1 and 2 times a week for an additional 4 weeks post-transplant.

3.2.4 In vitro proliferation assays

In vitro proliferation of lymphocytes from tolerant B6 mice were performed by combining $5x10^5$ spleen cells from tolerant B6 mice with Concanavalin A (ConA; 10 μg/ml, Sigma) or with 3×10^5 irradiated (2,500 rad) spleen cells from naïve BALB/c mice, WF rats, from the same (first party) or different (second party) neonatal porcine donors used in B6 mice. The cells were cultured in 96-well flat-bottom plates in a total volume of 0.2 ml of Eagle's modified essential medium (EMEM, Invitrogen, Burlington, ON, Canada) supplemented with 10% FBS (Invitrogen), 1×10^{-5} mol/l 2-mercaptoethanol, 2 x

10^{-3} mol/l L-glutamine and 1% antibiotics in triplicate at 37°C, 5% CO_2 and 95% air (14). After three, 4, and 5 days of culture T cell proliferation was determined by pulsing the cells of primary culture with one μCi [^{3}H]-thymidine/well for 18 h. Cells were then harvested onto glass microfiber filters (Wallac, Turku, Finland), and counts per minute (cpm) per sample were detected using the Wallac MicroBeta® TriLux luminescence counter (PerkinElmer, Waltham, MA, USA). *In vitro* proliferation of lymphocytes from naïve control B6 mice were detected after combining with the same stimulators.

3.2.5 Adoptive transfer studies

Streptozotocin-induced diabetic B6 *rag-/-* mice were transplanted with 500 islets from BALB/c mice or WF rats, or 2,000 islets from first party or second party neonatal porcine. At 150 days post-transplantation, all normoglycemic mice received intraperitoneal injection of $50x10^6$ spleen cells in 200 μl PBS from tolerant B6 mice or from naive non-transplanted B6 mice. Blood glucose levels of these mice were monitored three times a week after cell injection. The graft-bearing kidneys were removed for histological analysis at the time of rejection or at 60 days post-cell transfer if mice remained normoglycemic. The blood glucose levels of the latter group of mice were monitored to confirm that normoglycemia was graft-dependent. The presence of adoptively transferred immune cells was confirmed by flow cytometry.

3.2.6 CFSE labeling of spleen cells

Spleen cells from tolerant B6 mice or naïve non-transplanted B6 mice were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE)

according to the manufacturer's protocol (Molecular Probes, Eugene, OR, USA). Briefly, ten million per ml of spleen cells from B6 mouse recipients of NPI that maintained long-term normoglycemia were suspended in sterile PBS and incubated with 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR, USA) in the dark with periodic agitation at 37°C for 10 min. Excess CFSE was quenched with 5% FBS (Sigma) and the cells were washed with PBS. Fifty million CFSE labeled spleen cells in 200 μl PBS were injected into B6 *rag-/-* mice with established allogeneic BALB/c mouse or xenogeneic WF rat, first or second party NPI at 150 days post-transplantation. On the day of rejection or at 60 days post-cell transfer, spleen cells from reconstituted B6 *rag-/-* mice were isolated and $1x10^6$ cells were incubated with PE-Cy5 conjugated anti-mouse TCRβ chain to determine the proliferative response of T cells in recipient mice. CFSE specific fluorescence histograms were created using a BD FACS Calibur flow cytometry machine (BD Biosciences Pharmingen, Mississauga, Ontario, Canada) by gating on $TCR\beta^+$ T cells.

3.2.7 Skin transplantation

Tolerant B6 mice were transplanted with full-thickness skin from allogeneic BALB/c mice or second party xenogeneic neonatal porcine. Skin grafts were inspected daily until the time of rejection, which is defined as necrosis of the skin grafts. In a parallel experiment, skin transplantation was also performed on naïve B6 mice and B6 *rag-/-* mice as positive and negative control groups, respectively.

3.2.8 Immunohistological analysis

The presence of insulin-producing beta cells and foxp3^{+} immune cells in the grafts were examined following our published protocol (4,8,10). Graft-bearing kidneys were harvested and fixed in 10% buffered formalin solution and embedded in paraffin, then 5 μm sections were stained with guinea pig anti-porcine insulin primary antibody (1:1,000 dilution; DAKO laboratories, Mississauga, Ontario, Canada) for 30 min, followed by the addition of biotinylated goat anti-guinea pig IgG secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA, USA). Avidin-biotin complex/horseradish peroxidase (ABC/HP; Vector Laboratories, Burlingame, CA, USA) and 3, 3-diaminobenzidinetetrahydrochloride (DAB; BioGenex, San Ramon, CA, USA) were used to detect positive cells (brown color). All paraffin sections were counter-stained with Harris' hematoxylin and eosin. The other half of the kidney was embedded in OCT compound (Miles Scientific, Naperville, IL, USA) and kept frozen at -80°C to identify the foxp3 positive cells in the grafts. Five-micron frozen sections of the graft were air dried for 10 min then fixed in acetone for 3 min at 4°C. Triton X-100 (0.1% in PBS) was added onto the sections and left for 10 min at room temperature, and then the sections were washed in PBS. Non-specific binding was eliminated by incubating the tissue sections in 2% fetal bovine serum (FBS) in PBS for 30 min. In addition, endogenous avidin and biotin or biotin-binding proteins present in the sections were also eliminated using the avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, USA). Rat anti-mouse foxp3 antibody (1:25 dilution; eBioscience, San Diego, CA, USA) was applied to designated tissue sections for 60 min at room temperature.

Biotinylated goat anti-rat IgG (1:200 dilution; Southern Biotechnology Associates, Inc., Birmingham, AL, USA) secondary antibody was added and incubated for 30 min. ABC/HP reagent (Vector Laboratories) and DAB were applied to produce a brown color. Sections were then counter-stained with Harris' hematoxylin Sections were counter-stained with Harris' hematoxylin.

3.2.9 Detection of anti-islet or anti-skin donor antibodies by flow cytometry

The humoral immune response of the recipients to the donor grafts was determined by measuring the levels of mouse anti-islet or anti-skin donor IgG antibodies from blood serum of each transplant recipient using flow cytometry (4,8,10). Peripheral blood samples from B6 or reconstituted B6 *rag-/-* mice that either rejected or accepted the grafts were collected, and sera were isolated. Spleen cells ($1x10^6$) obtained from the donors were incubated with serum from recipients of WF rat islets or pig islet and/or skin (1:128 dilution) or BALB/c mouse islets and skin recipients (1:64 dilution) for 1 hour at 37°C, 5% CO_2, and 95% air. Spleen cells were then washed with PBS and incubated for 1 hour at 4^oC with fluorescein isothiocyanate (FITC)-conjugated rat adsorbed goat anti-mouse IgG antibody (1:200 dilution, Southern Biotechnology Associates, Inc. Birmingham, Alabama, AL, USA) to detect the levels of mouse anti-porcine or mouse anti-rat IgG antibody. FITC-conjugated affinity pure $F(ab)_2$ fragment rabbit anti-mouse IgG, Fcγ fragment specific antibody (1:100 dilution, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was used to detect the levels of mouse anti-mouse IgG antibody. The percentage of cells bound to the antibody was determined from single parameter fluorescence histograms on a

BD FACS Calibur flow cytometry machine after gating on viable lymphocytes. Controls for this experiment include unstained spleen cells, and spleen cells incubated with secondary antibody alone without mouse serum.

3.2.10 Characterization of lymphocytes by flow cytometry

Spleen cells from reconstituted B6 *rag-/-* mouse recipients were isolated on the day of rejection or at >60 days post-cell transfer. Spleen cells were isolated by mechanical disruption of spleen tissue between rough edges of glass slides. Erythrocytes from spleen cell suspension were depleted using red blood cell lyses buffer. Absolute lymphocyte numbers were determined by staining the cells with Trypan blue exclusion dye, and live cells were counted using a hemocytometer (14). One million spleen cells were incubated with fluorescent conjugated antibodies (1:100 dilution, eBioscience) for 30 min at 4^{o}C. Spleen cells were washed two times with PBS and suspended in 300 μl of FACS buffer (2% FBS in PBS). Fluorescence histograms were created using a BD FACS Calibur flow cytometry machine and were used to determine the percentage of positive cells labeled with the corresponding antibodies. Controls for this experiment include unstained spleen cells from the corresponding mice and spleen cells from non-transplanted naïve B6 mice that were either not incubated or incubated with the antibodies.

3.2.11 Statistical analysis

Statistical differences between groups in mixed lymphocyte culture assays and mouse anti-donor IgG antibody levels were determined using non-parametric Mann-Whitney U test. Statistical differences in graft survival among the groups

were analyzed using the Kaplan-Meier Log rank test. All statistical tests were performed using SPSS statistical software, version 13.0 for Windows (Chicago, IL, USA). A p value of < 0.05 was considered to be statistically significant.

3.3 RESULTS

3.3.1 Combination of anti-LFA-1 and anti-CD154 mAbs induces porcine specific unresponsiveness in vitro.

The specificity of tolerance induced by the combined anti-LFA-1 and anti-CD154 mAbs was examined in both *in vitro* proliferation assays and *in vivo* adoptive transfer of cells from tolerant B6 mouse recipients of NPI xenografts. On days 3, 4 and 5 of culture, lymphocytes from spleen of tolerant B6 mice responded robustly after stimulation with allogeneic BALB/c mouse (92,531 ± 8,044, 49,892 ± 5,906, and 24,236 ± 2,929 cpm, respectively) or xenogeneic WF rat spleen cells (91,933 ± 2,815, 61,788 ± 4,161, and 28,647 ± 2,876 cpm, respectively, Figure 3.1A). These responses were comparable to the proliferation of lymphocytes from spleen of naïve non-transplanted B6 mice stimulated with the same allogeneic BALB/c mouse (100,136 ± 7,775, 53,748 ± 5,857, and 24,854 ± 3,688 cpm, respectively) and xenogeneic WF rat spleen cells (94,662 ± 2,647, 65,769 ± 4,507, and 28,350 ± 1,890, respectively, Figure 3.1B) on days 3, 4 and 5 of culture.

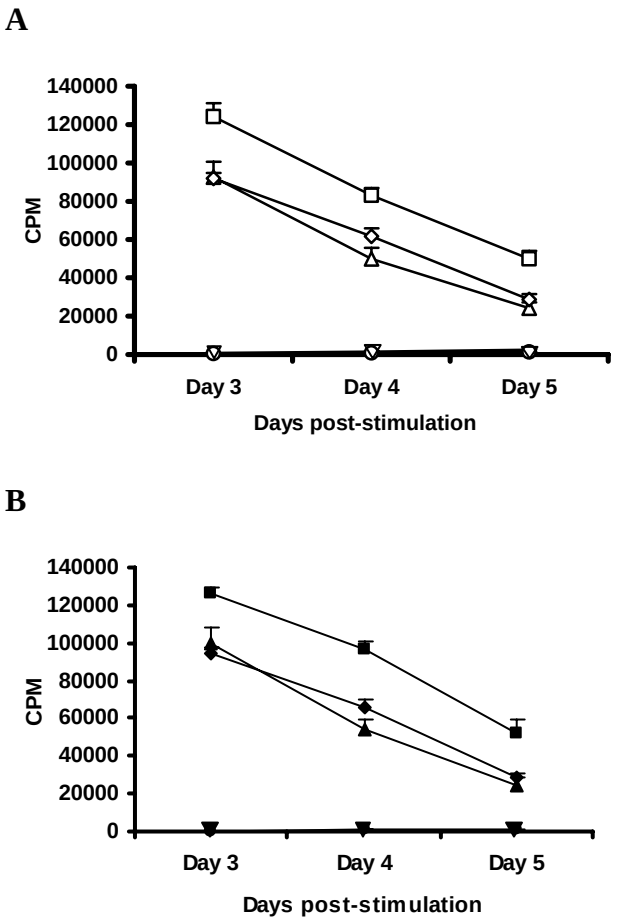

Figure 3.1: Lymphocytes from tolerant B6 mice showed a porcine specific unresponsiveness *in vitro*. Lack of detectable proliferation was observed when lymphocytes from tolerant (A, white circles and inverted triangles, n=3 in each group) and naïve non-transplanted B6 mice (B, black circles and inverted triangles, n=3 in each group) were stimulated with spleen cells from first or second party porcine donors, respectively. In contrast, lymphocytes from tolerant B6 mice (A) responded robustly after stimulation with ConA (white squares), allogeneic BALB/c mouse (white triangles) or xenogeneic WF rat spleen cells (white diamonds). These results were comparable to what was observed in the responses of lymphocytes from naïve non-transplanted B6 mice (B) when co-cultured with the same stimulators (black squares for ConA, black triangles for BALB/c mouse and black diamonds for WF rat spleen cells).

In contrast, no measurable proliferation of lymphocytes from tolerant B6 mouse recipients of NPI were observed after stimulation with first party (560 ± 128, 758 ± 98, and 1,291 ± 239 cpm, respectively) or second party (765 ± 77, 1,570 ± 58, and 2,458 ± 216 cpm, respectively) porcine donors on 3, 4 and 5 days of culture (Figure 3.1A). The responses observed in this group was comparable to those seen in lymphocytes from spleen of naïve non-transplanted B6 mice stimulated with the same cell populations on the same days of culture (465 ± 31, 656 ± 34, and 1,068 ± 87, respectively, Figure 3.1B).

3.3.2 Combination of anti-LFA-1 and anti-CD154 mAbs induces porcine specific unresponsiveness in vivo.

All of the B6 *rag-/-* mice transplanted with islets, achieved and maintained normal blood glucose levels for more than 150 days post-transplantation (Figure 3.2). Those that received allogeneic BALB/c mouse or xenogeneic WF rat islets achieved normoglycemia within 1 day post-transplantation (Figure 3.2A and B, respectively) while those transplanted with first or second party xenogeneic NPI achieved normoglycemia at 78 days post-transplantation (Figure 3.2C and D, respectively).

B6 *rag-/-* mice with established allogeneic mouse islet grafts that were reconstituted with spleen cells from tolerant B6 mouse recipients of first party NPI became diabetic within 14 and 25 days post-cell injection with a mean survival time (MST) of 20.3 ± 1.9 days post-cell injection (Table 3.1 and Figure 3.2A).

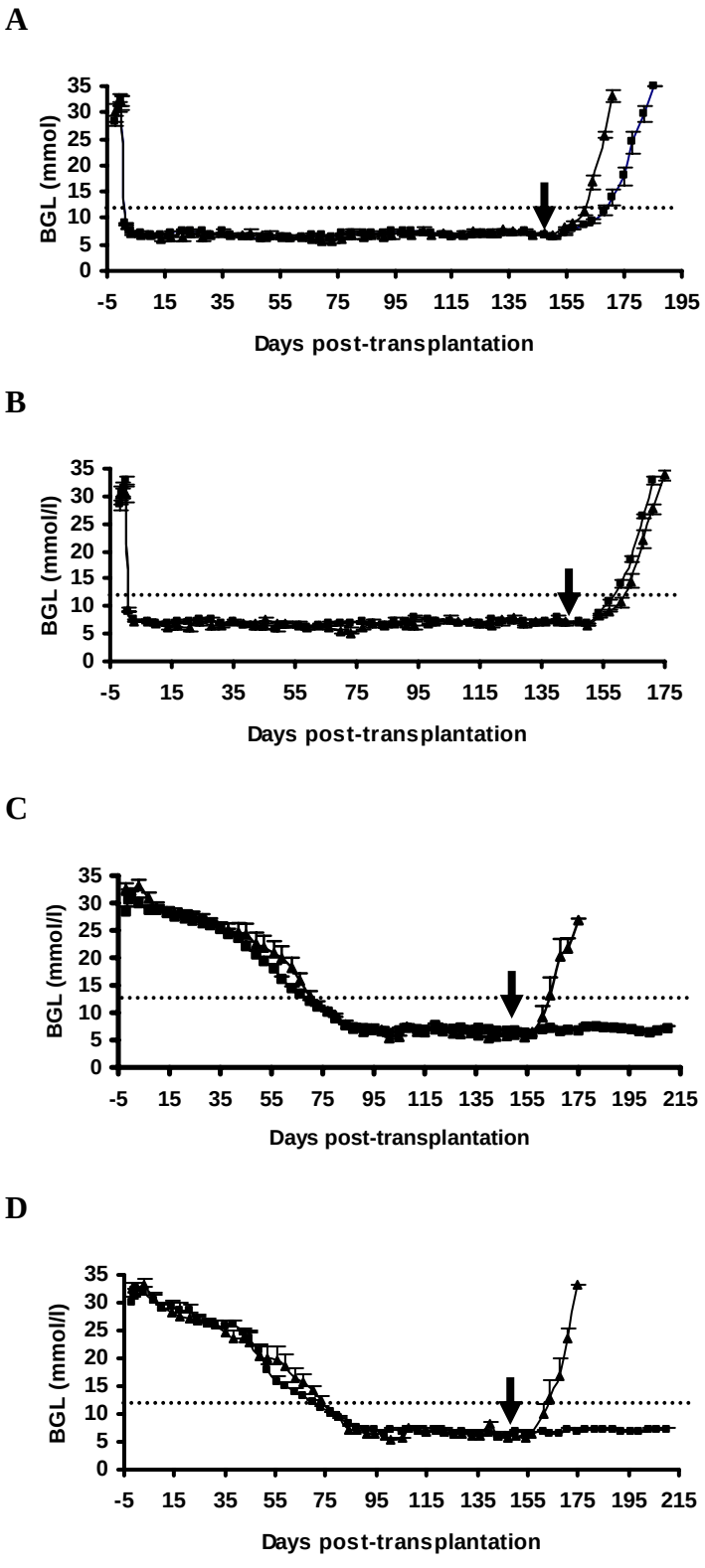

Figure 3.2: Lymphocytes from tolerant B6 mice showed a porcine specific unresponsiveness *in vivo*. Metabolic follow-up of B6 *rag-/-* mouse recipients of

allogeneic BALB/c mouse or concordant WF rat islets (n=12 in each group) showed that these mice achieved normoglycemia within 1 day after transplantation (A and B, respectively). While mice transplanted with first (C) or second (D) party xenogeneic neonatal porcine islets (n=13 in each group) achieved normoglycemia at 78 days post-transplantation demonstrating the immature nature of the islets. All B6 *rag-/-* mice with established allogeneic BALB/c mouse islet grafts that were reconstituted with $50x10^6$ spleen cells from tolerant (black squares, n=7) or naive non-transplanted B6 mice (black triangles, n=5) became diabetic within 9 and 25 days post-cell injection. Similarly all B6 *rag-/-* mice with established xenogeneic WF rat islet grafts (B) reconstituted with $50x10^6$ spleen cells from tolerant (black squares, n=7) or naive non-transplanted (black triangles, n=5) B6 mice became diabetic within 8 and 15 days post-cell injection. In contrast, reconstituted B6 *rag-/-* mouse recipients of first (C, black squares, n=9) and second (D, black squares, n=10) party NPI xenografts with spleen cells from tolerant B6 mice with first party NPI xenografts maintained normal blood glucose levels until the end of the study. On the contrary, all B6 *rag-/-* mouse recipients of first (C, black triangles, n=4) and second (D, black triangles, n=3) party NPI xenografts became diabetic at day 15 after receiving spleen cells from naïve non-transplanted B6 mice.

Those that were with spleen cells from naïve non-transplanted B6 mice also became diabetic between 9 and 14 days post-cell injection (MST is 11.6 ± 0.8 days, Table 3.1 and Figure 3.2A). The difference in the rate of rejection between these two groups was found to be statistically significant ($p<0.002$). Similarly, B6 *rag-/-* mice transplanted with rat islets and reconstituted with spleen cells from tolerant B6 mice with NPI xenografts rejected their grafts by 9.6 ± 0.6 days post-cell injection (Table 3.1 and Figure 3.2B). Those that received spleen cells from naïve non-transplanted B6 mice also rejected their grafts between 10 and 15 days with a MST of 12.0 ± 0.8 days post-cell injection (Table 3.1 and Figure 3.2B). In contrast, B6 *rag-/-* mice with first or second party NPI xenografts that were injected with spleen cells from tolerant B6 mice with first party NPI xenografts maintained normal blood glucose levels until the end of the study (>60 days post-cell injection, Table 3.1, Figure 3.2C and D, respectively). This indicates that

cells from B6 mice tolerant to first party NPI xenografts are also unresponsive to the second party NPI xenografts. On the contrary, all B6 *rag-/-* mouse recipients of first or second party NPI xenografts rejected their grafts by 12.3 ± 1.1 or 13.0 ± 1.2 days after receiving spleen cells from naïve non-transplanted B6 mice, respectively (Table 3.1, Figure 3.2C and D).

Table 3.1: Graft survival of allogeneic BALB/c mouse islets, xenogeneic WF rat islets, first party and second party NPI xenografts in B6 *rag -/-* mice injected with spleen cells from either tolerant B6 mouse recipients of first party NPI xenografts or naive non-transplanted B6 mice.

Source of Islets	Source of Spleen Cells	n	Graft Survival (days post-cell injection)	Mean Survival Time (MST) ± SEM
BALB/c mouse	Tolerant B6	7	14, 15, 17, 21, 25(x3)	20.3 ± 1.9*
BALB/c mouse	Naive B6	5	9, 11, 12(x2), 14	11.6 ± 0.8*
WF rat	Tolerant B6	7	8 (x3), 10, 11(x3)	9.6 ± 0.6
WF rat	Naive B6	5	10, 11, 12 (x2), 15	12.0 ± 0.8
First party NPI	Tolerant B6	9	>60(x9)	>60†
Second party NPI	Tolerant B6	10	>60(x10)	>60†
First party NPI	Naive B6	4	10, 11, 13, 15	12.3 ± 1.1
Second party NPI	Naive B6	3	11, 13, 15	13.0 ± 1.2

* $p<0.002$ between groups, †$p<0.05$ vs. other groups which just received naive cells,

Mononuclear cell infiltrate and no islets were detected in the graft site of B6 *rag-/-* mice transplanted with BALB/c mouse (Figure 3.3A) or WF rat islets (Figure 3.3B).

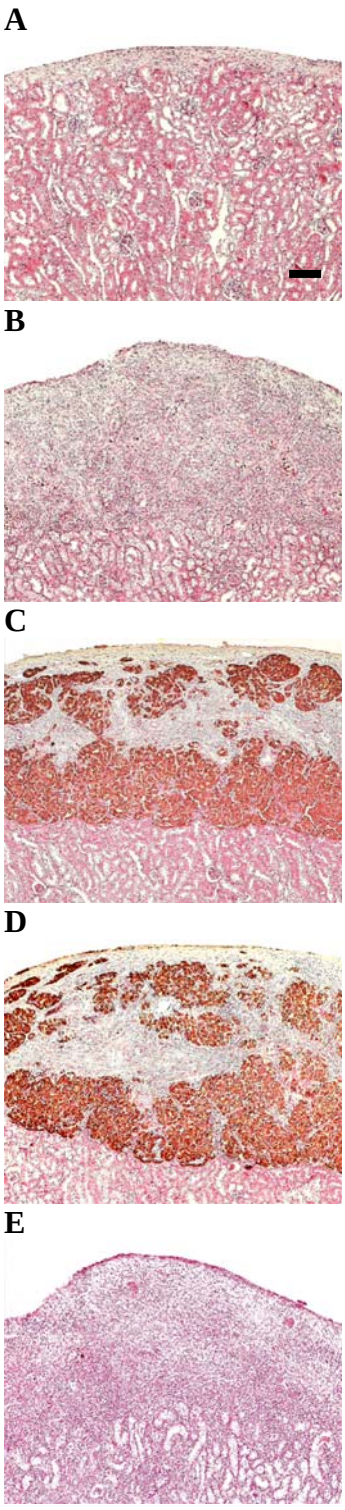

Figure 3.3: NPI xenografts but not mouse islet allografts and rat islet xenografts in B6 *rag-/-* mouse recipients reconstituted with spleen cells from tolerant B6 mice remained intact with abundant insulin-positive cells. Representative allogeneic BALB/c mouse (A), xenogeneic WF rat (B) islet grafts from B6 *rag-/-* mice injected with lymphocytes from tolerant B6 mice had no

insulin positive cells but contain mononuclear cell infiltrate. NPI xenografts from B6 *rag-/-* mice with established first (C) and second party (D) NPI xenografts reconstituted with spleen cells from B6 mice tolerant to first party NPI xenografts showed intact islets staining strongly positive for insulin. In contrast, the first and second party NPI xenografts from B6 *rag-/-* mice reconstituted with spleen cells from naïve non-transplanted B6 mice had no insulin positive cells but contained mononuclear cell infiltrate (E). Scale bar represents 100 μm.

In contrast, the islet grafts from B6 *rag-/-* mice with established first (Figure 3.3C) and second party (Figure 3.3D) NPI xenografts reconstituted with spleen cells from B6 mice tolerant to first party NPI xenografts showed intact islets staining strongly positive for insulin. While the grafts from B6 *rag-/-* mice with established first or second party NPI xenografts that were reconstituted with spleen cells from naïve non-transplanted B6 mice contained mononuclear cell infiltrate and no insulin positive cells (Figure 3.3E).

The levels of allogeneic anti-mouse (38.8% - 45.2%, n=7, Figure 3.4A) and xenogeneic anti-rat (45.3% - 53.7%, n=7, Figure 3.4B) IgG antibodies detected in the B6 *rag-/-* mice with allogeneic mouse and xenogeneic rat islets, respectively reconstituted with tolerant B6 spleen cells were significantly higher than the levels of anti-porcine IgG antibodies in the B6 *rag-/-* mice with first (5.3% - 7.5%, n=9, Figure 3.4C) and second (5.0 % - 7.8%, n=10, Figure 3.4D) party NPI xenografts reconstituted with same spleen cells ($p<0.003$). In addition the level of anti-porcine IgG antibodies detected in the B6 *rag-/-* mouse recipients of first or second party NPI that received spleen cells from tolerant B6 mice were significantly lower ($p<0.003$) than those observed in B6 *rag-/-* mouse recipients of NPI that received spleen cells from naïve non-transplanted B6 mice (58.3% - 68.1%, n=5, Figure 3.4E).

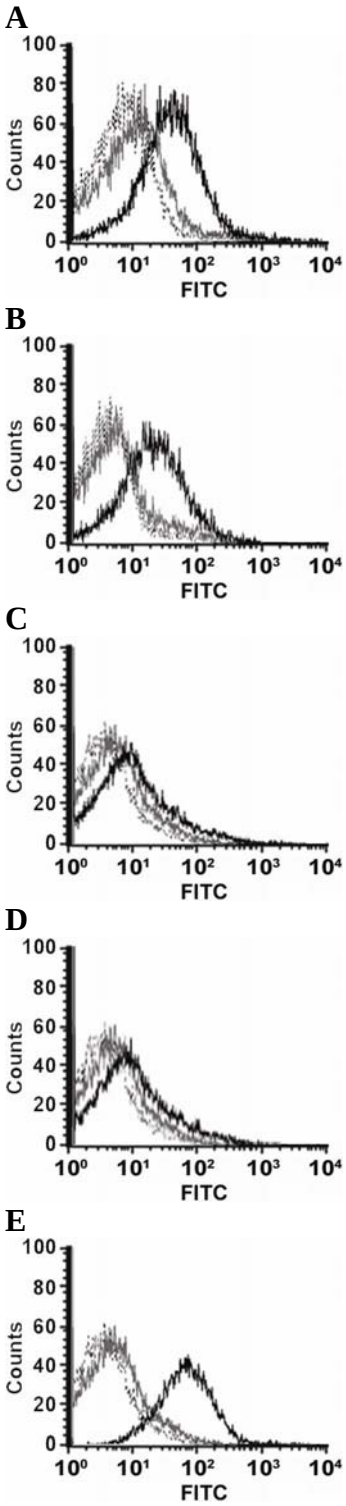

Figure 3.4: Humoral responses of tolerant lymphocytes to the allogeneic mouse and xenogeneic rat and porcine islets *in vivo.* The levels of anti-porcine IgG antibodies from reconstituted B6 *rag-/-* mouse recipients of allogeneic (38.8% - 45.2%, n=7, A) and xenogeneic rat (45.3% - 53.7%, n=7, B) islets were higher than those detected in recipients of first (5.3% - 7.5%, n=9, C) and second

(5.0 % - 7.8%, n=10, D) party NPI xenografts. The levels of anti-porcine IgG antibodies observed in B6 *rag-/-* mouse recipients of NPI that received spleen cells from naïve non-transplanted B6 mice were also significantly higher (58.3% - 68.1%, n=7, E) than those observed in mice reconstituted with spleen cells from tolerant B6 mice. Representative histograms are shown and controls for this experiment consisted of unstained (dashed black line) and secondary antibody without serum (solid gray line).

CFSE labeling of spleen cells from tolerant B6 mice revealed that T cells from tolerant B6 mice when injected into B6 *rag-/-* mice with established allogeneic mouse (Figure 3.5A) or xenogeneic rat (Figure 3.5B) islet grafts proliferate robustly. The pattern of proliferation was similar to what was observed when T cells from naïve non-transplanted B6 mice were injected into B6 *rag-/-* mice with established first or second party NPI xenografts (Figure 3.5E). In contrast, a large proportion of T cells from B6 mice tolerant to first party NPI xenografts did not proliferate robustly after injection into B6 *rag-/-* mice with established first and second party NPI xenografts (Figure 3.5C and D, respectively). However, in the absence of NPI xenografts, these cells were able to proliferate when injected into naïve non-transplanted B6 *rag-/-* mice (Figure 3.5F).

After rejection of the islet grafts has been established or at 60 days post-cell injection if reconstituted B6 *rag-/-* mice remained normoglycemic, the immune cells from B6 *rag-/-* mice were recovered and examined by flow cytometry. We found that the total number of immune cells from B6 *rag-/-* mice with allogeneic BALB/c mouse or xenogeneic WF rat islet grafts were not significantly different when these mice were reconstituted with spleen cells from either tolerant or naïve non-transplanted B6 mice (Figure 3.6A).

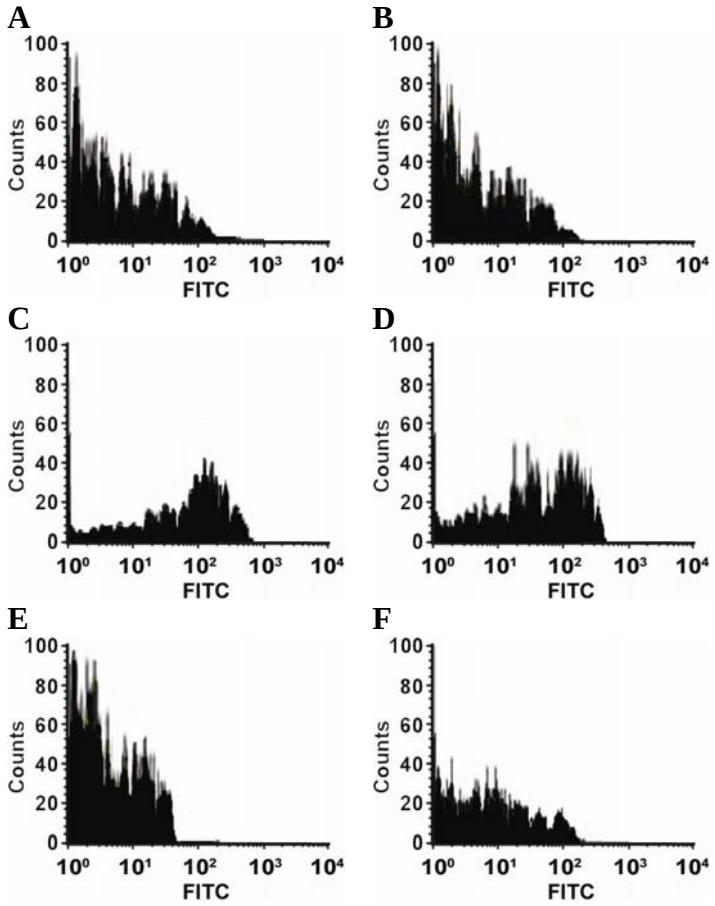

Figure 3.5: Adoptively transferred lymphocytes from tolerant B6 mice showed a limited porcine-specific proliferation *in vivo*. CFSE labeled spleen cells from tolerant B6 mice injected into B6 *rag-/-* mouse recipients that rejected the allogeneic BALB/c mouse or xenogeneic WF rat islet grafts, showed robust T cell proliferation (A and B, respectively). The pattern of proliferation was similar to what was observed when T cells from naïve non-transplanted B6 mice were injected into B6 *rag-/-* mice with established first or second party NPI xenografts (E). In contrast, CFSE labeled T cells from B6 mice tolerant to first party NPI xenografts showed that a large proportion of these cells did not proliferate robustly after injection into B6 *rag-/-* mice with established first (C) and second (D) party NPI xenografts. However, in the absence of NPI xenografts, these cells were able to proliferate when injected into naïve B6 *rag-/-* mice (F).

In contrast, we found a significant ($p<0.05$) difference between the total numbers of immune cells recovered from B6 *rag-/-* mice with first and second party NPI xenografts that received spleen cells from tolerant B6 mice compared to those that received spleen cells from naïve non-transplanted B6 mice. Similarly,

despite the observed proliferation of immune cells from tolerant B6 mice after reconstitution in naïve non-transplanted B6 *rag-/-* mice, the total number of immune cells in these mice was significantly ($p<0.05$) lower than those observed in naïve non-transplanted B6 *rag-/-* mice that received spleen cells from naïve non-transplanted B6 mice (Figure 3.6A). Further characterization of immune cells show that the frequencies of $CD4^+$ (Figure 3.6B) and $CD8^+$ (Figure 3.6C) T cells in B6 *rag-/-* mouse recipients of first and second party NPI xenografts were significantly ($p<0.05$) less than those detected in B6 *rag-/-* mice that received spleen cells from naïve non-transplanted B6 mice. These results correspond with the pattern of *in vivo* proliferation of CFSE-labeled immune cells seen in these mice (Figure 3.5C - E, respectively). We also found a significant ($p<0.05$) decrease in $CD19^+$ B cell population in B6 *rag-/-* mouse recipients of NPI after 60 days post-reconstitution of tolerant immune cells compared to the same group of NPI recipients reconstituted with spleen cells from naïve non-transplanted B6 mice (Figure 3.6D). Taken together, these results show that tolerance induced by short-term administrations of anti-LFA-1 and anti-CD154 mAbs is specific to the porcine species.

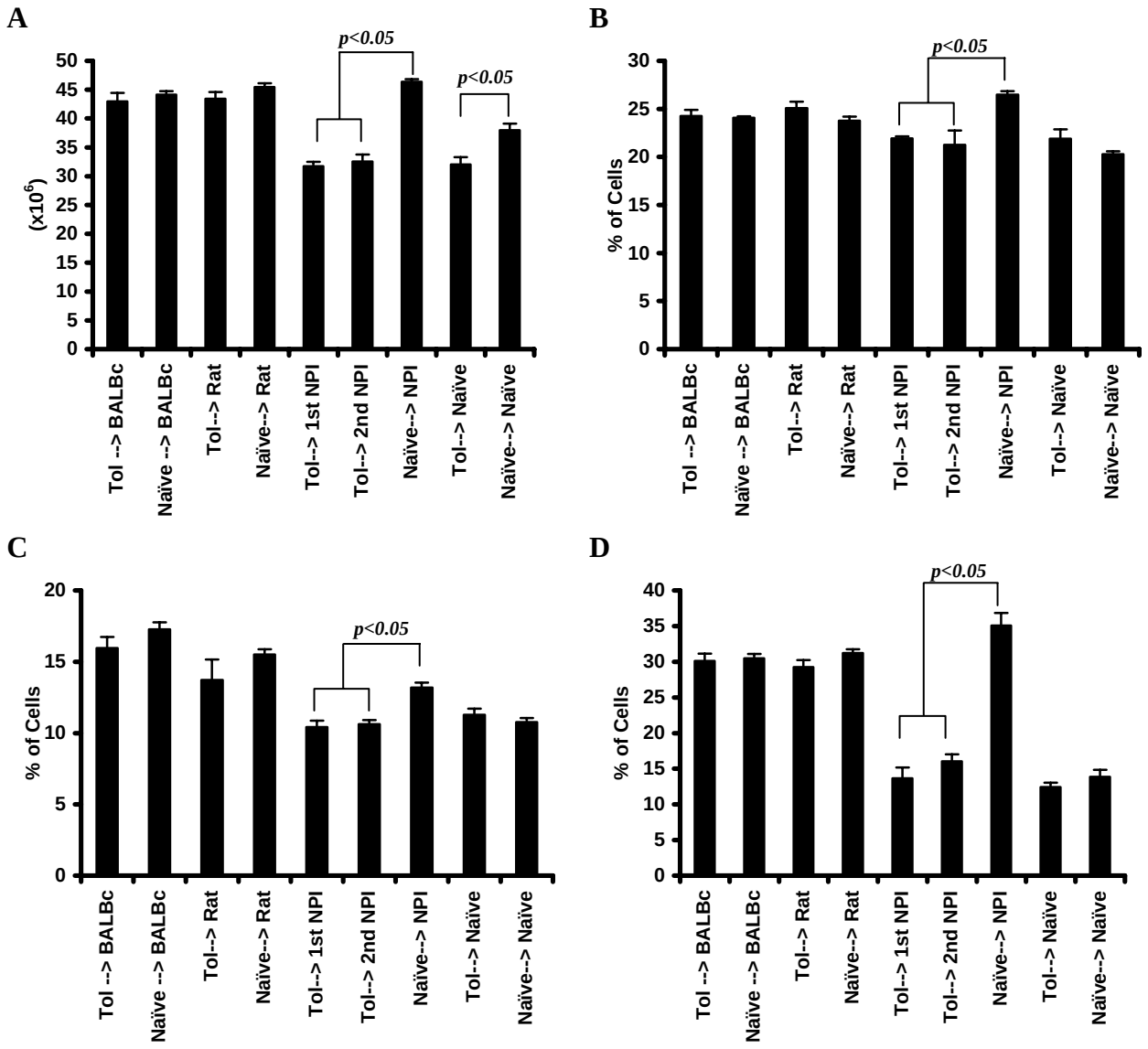

Figure 3.6: Immune cells from tolerant or naïve non-transplanted B6 mice after reconstitution into B6 *rag-/-* mouse recipients of allogeneic BALB/c mouse islets, xenogeneic WF rat islets, first or second party NPI. The total number of immune cells recovered from B6 *rag-/-* mice with allogeneic or xenogeneic rat islet grafts reconstituted with spleen cells from either tolerant or naïve non-transplanted B6 mice were not significantly different (A). In contrast, a significant ($p<0.05$) difference was observed between the total numbers of immune cells recovered from B6 *rag-/-* mice with first and second party NPI xenografts that received spleen cells from tolerant B6 mice compared to those that received spleen cells from naïve non-transplanted B6 mice (A). Similarly, the total number of tolerant immune cells recovered in naïve non-transplanted B6 *rag-/-* mice was significantly ($p<0.05$) lower than those observed in naïve non-transplanted B6 *rag-/-* mice that received spleen cells from naïve non-transplanted B6 mice (A). Further characterization of immune cells show that the frequencies

of $CD4^+$ (B) and $CD8^+$ (C) T cells in B6 *rag-/-* mouse recipients of first and second party NPI xenografts were significantly less than those detected in B6 *rag-/-* mice that received spleen cells from naïve non-transplanted B6 mice ($p<0.05$). Also a significant ($p<0.05$) decrease in $CD19^+$ B cell population in B6 *rag-/-* mouse recipients of NPI was detected after 60 days post-cell transfer of immune cells from tolerant B6 mice compared to the same group of NPI recipients reconstituted with spleen cells from naïve non-transplanted B6 mice (D).

3.3.3 Tolerance induced by combination of anti-LFA-1 and anti-CD154 mAbs is specific to porcine islet but not to porcine skin xenografts.

All B6 mice treated with combined anti-LFA-1 and anti-CD154 mAbs achieved and maintained normal blood glucose levels for more than 100 days post-transplantation (Figure 3.7A and B). However, these mice rejected the second party porcine skin xenografts by 15.8 ± 1.7 days post-transplantation (Table 3.2). All untreated B6 mice transplanted with the same porcine skin rejected their grafts significantly faster (6.8 ± 0.7 days post-transplantation, $p<0.002$, Table 3.2) compared to the rate of rejection observed in tolerant B6 mice. While all B6 *rag-/-* mice transplanted with the second party porcine skin maintained their skin grafts until the end of the study (>60 days post-transplantation, Table 3.2). We also found that all tolerant B6 mice transplanted with full-thickness allogeneic BALB/c skin rejected their grafts by 10.8 ± 0.7 days post-transplantation similar to what was observed in untreated B6 mice (10.6 ± 0.8 days post-transplantation, Table 3.2). Despite the rejection of second party porcine and allogeneic BALB/c mouse skin grafts, tolerant B6 mice remained normoglycemic until the end of the study (>200 days post-transplantation, Figure 3.7A and B, respectively) and they only became diabetic when the NPI xenografts were removed.

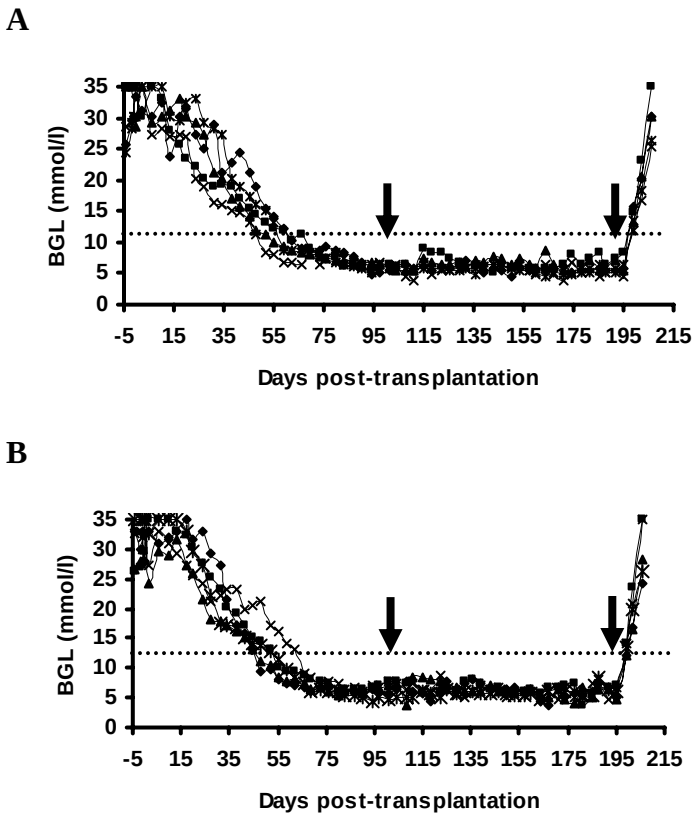

Figure 3.7: Tolerance provided by combined anti-LFA-1 and anti-CD154 mAb therapy to porcine islets could not be extended to skin grafts. All tolerant B6 mice transplanted with second party porcine (A) and allogeneic BALB/c mouse (B) skin grafts (first arrow) remained normoglycemic until after removal of the left kidney bearing the first party NPI xenograft (second arrow).

Histological analysis of the NPI xenografts from these mice showed intact islets (Figure 3.8A and B) with immune cells expressing foxp3 that were surrounding but not infiltrating the NPI xenografts (Figure 3.8C and D).

Table 3.2: Graft survival of allogeneic BALB/c mouse or second party porcine skin grafts transplanted in tolerant B6 mouse recipients of first party NPI xenografts, naive non-transplanted B6 or naive non-transplanted B6 *rag-/-* mice.

Skin graft recipient	Skin type	n	Graft survival (days post-transplantation)	Mean Survival Time (MST) ± SEM
Tolerant B6	Porcine	5	11, 14, 16, 17, 21	15.8 ± 1.7*
Naïve B6	Porcine	5	5, 6, 7(x2), 9	6.8 ± 0.7*
Naïve B6 *rag-/-*	Porcine	3	>60 (3)	>60
Tolerant B6	BALB/c	5	9, 10, 11(x2), 13	10.8 ± 0.7
Naïve B6	BALB/c	5	8, 10, 11, 12 (x2)	10.6 ± 0.8
Naïve B6 *rag-/-*	BALB/c	3	>60 (x3)	>60

* $p<0.002$ between the groups

Despite the rejection of second party porcine skin grafts, the levels of IgG antibodies against the second party porcine skin donors were significantly lower (14.3% - 20.2%, n=5, $p<0.01$, Figure 3.9A) compared to the levels of IgG antibodies (57.3% - 64.1%, n=5) against the same porcine skin xenografts in untreated B6 mice (Figure 3.9B). The levels of allogeneic anti-mouse IgG antibodies (Figure 3.9C) in tolerant B6 mice that rejected the allogeneic BALB/c mouse skin grafts were comparable to those detected in B6 mice that rejected the same allogeneic mouse skin grafts (Figure 3.9D).

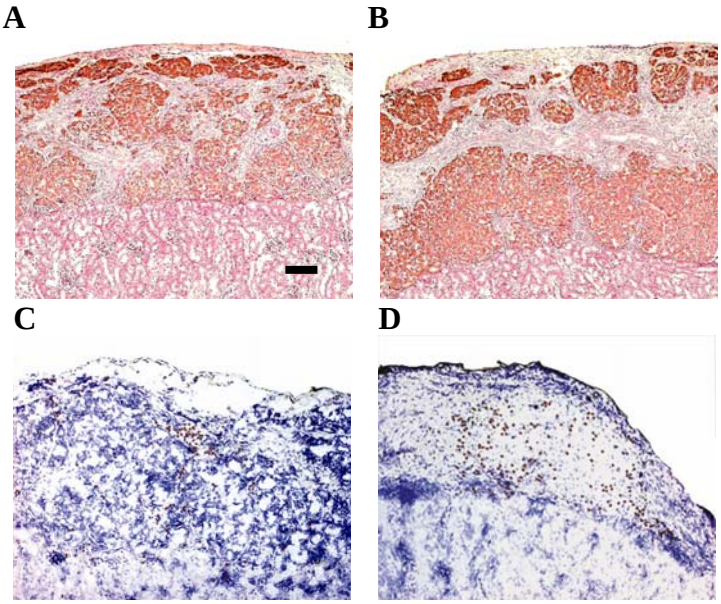

Figure 3.8: Histological analysis of the NPI xenografts harvested from the tolerant B6 mouse recipients of allogeneic mouse or xenogeneic porcine skin grafts. Representative first party NPI xenografts from tolerant B6 mice with second party porcine (A) or allogeneic BALB/c mouse (B) skin grafts had intact islets composed of abundant insulin positive cells as well as foxp3^{+} cells (C and D, respectively). Scale bar represents 100 μm.

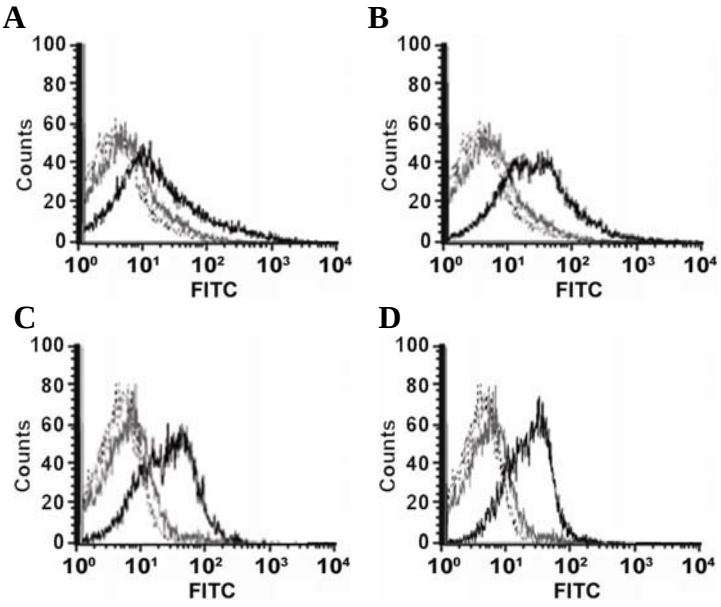

Figure 3.9: Humoral responses of tolerant B6 lymphocytes to allogeneic mouse and second party xenogeneic porcine skin donors. The levels of IgG antibodies against the second party porcine skin donors are significantly lower (14.3%-20.2%, n=5, $p<0.01$, A) compared to the levels of IgG antibodies against

the same porcine skin xenografts in untreated B6 mice (57.3%-64.1%, n=5, B). However, the levels of allogeneic anti-mouse IgG antibodies in tolerant mouse recipients of allogeneic BALB/c skin (43.6%-59.8%, n=5, C) were comparable to the levels of IgG antibodies detected in naive B6 mouse recipients of BALB/c skin that rejected the same allogeneic mouse skin grafts (44.3%-61.7%, n=5, D). Representative histograms are shown and controls for this experiment consisted of unstained (dashed black line) and secondary antibody without serum (solid gray line).

Taken together, these results show that tolerance induced by combined anti-LFA-1 and anti-CD154 mAb therapy is specific to porcine islet xenografts but not to porcine skin xenografts and that, this tolerance could not be broken even when xenogeneic porcine or allogeneic mouse skin grafts were rejected.

3.4 DISCUSSION

Our previous studies demonstrated that transient therapies directed against T lymphocyte activation and function using mAbs resulted in long-term islet xenograft survival, especially regarding highly phylogenetically-disparate xenograft donors (4,5,8,10). In particular, we recently showed that short-term administrations of a combination of anti-LFA-1 and anti-CD154 mAb therapy resulted in tolerance to NPI xenografts that is mediated by T regulatory cells (8).

In the current study, we demonstrated that tolerance induced by this combined mAb therapy is specific to porcine islets from either first or second party donors. Similar to what we found, species-specific tolerance to islet xenografts was also reported earlier by other groups (15-17). For example, Goss et al (18) showed that 50% of recipients that were treated with anti-lymphocyte serum (ALS) did not reject their third party Lewis rat islet xenografts. Similarly, Gordon et al (19) demonstrated that the combination of donor-specific transfusion

(DST) and anti-CD154 mAb induces species-specific prolongation of rat islet xenografts. Lehnert et al (20) also demonstrated that a combination of anti-CD154 mAb with CTLA4Fc produced indefinite survival of rat islet xenografts in 100% of mouse recipients. It is possible that rat or porcine of different strains share a wide range of species-specific antigens that are recognized through indirect antigen presentation in the mouse host. Thus, tolerance induced to the repertoire of indirect presented xenograft-associated antigens may demonstrate high degree of cross-reactivity with unrelated donors (21). It is interesting to note that while islet allograft rejection has both MHC class II–dependent and –independent mechanisms of rejection, the response to porcine xenografts appears to be greatly reliant on the indirect MHC class II–restricted pathway. Despite being finite in nature, the indirect anti-xenograft response appears to be extremely virulent but once tolerance to the islet xenografts is achieved, the state of unresponsiveness appears to be universal to the species.

Our results indicate that maintenance of tolerance induced by combined anti-LFA-1 and anti-CD154 mAbs requires the presence of porcine antigens responsible for induction of tolerance and suggest for a perpetual role for host antigen-presenting cells (APCs) in maintaining antigen exposure to T regulatory cells (22,23). Similar to what was previously demonstrated, we showed that antigen-specific suppression can be adoptively transferred (24,25) as was found in our adoptive transfer experiments where suppression activity of T regulatory cells have been observed in B6 *rag-/-* mouse recipients of either first or second party NPI xenografts. It is possible that due to the lack of porcine -specific antigens in

BALB/c mouse or WF rat islet grafts, the T regulatory cells were not activated and suppression did not take place. Therefore, the antigen-specificity observed both *in vitro* and *in vivo* could be explained by the hypothesis that T regulatory cells are activated only in hosts that has the antigens for which they are specific.

Unlike the previous study by Gordon et al (26), which showed that combined DST with anti-CD154 mAb therapy could be effective in prolonging the survival of rat skin xenografts we showed that tolerance to porcine islets induced by combined anti-LFA-1 and anti-CD154 mAbs could not be extended to porcine skin xenografts. The rejection of skin may require different co-stimulation pathways or additional pathways for T cell activation. Gordon et al (27) suggested that such pathways might be mediated by APCs found in skin but not in islets, an example would be the epidermal Langerhans cells. Our results suggest that tolerance to NPI xenografts induced by short-term administrations of combined anti-LFA-1 and anti-CD154 mAbs is specific to antigens that are common to porcine islets but not to porcine skin. In addition, all of the tolerant B6 mice re-transplanted with second party allogeneic BALB/c skin or xenogeneic porcine skin maintained normoglycemia showing that tolerance to NPI xenografts could not be broken during the rejection process of mouse or porcine skin xenografts. We sought evidence of T regulatory cells in the islet graft sites of these mice and showed the presence of foxp3^{+} cells surrounding the protected NPI xenografts. This finding suggests that cell-mediated regulation, at least in part, might be operating within the tolerated islet grafts. As was suggested, foxp3^{+} T cells in the graft play a key role in dominant tolerance induced by combined DST

and anti-CD154 mAb (28). It is possible that T regulatory cells in NPI xenograft site create a state of acquired immunological privilege site by which tissues are able to resist potentially damaging immune reactions during the rejection process of allogeneic BALB/c mouse or xenogeneic porcine skin grafts. Such an idea has recently been advocated for how tumors may avoid immunological control (29). A significant delay however, in the rejection of porcine skin xenografts in tolerant B6 mice compared to the rejection of the same porcine skin grafts in untreated B6 mouse recipients was observed which could be partially due to the expression of some porcine antigens that may be common between the porcine islets and porcine skin. It remains to be determined whether tolerance to NPI xenografts induced by combined anti-LFA-1 and anti-CD154 mAbs is dominant.

In conclusion, the data presented here indicate that tolerance induced by short-term administrations of a combination of anti-LFA-1 and anti-CD154 mAbs is species- and tissue- specific. Our results suggest the possibility of development of clinical protocols using biologic agents that simultaneously target both adhesion and co-stimulation pathways for preventing rejection and induction of tolerance to NPI xenografts.

3.5 REFERENCES

1. Ryan,EA, Paty,BW, Senior,PA, Bigam,D, Alfadhli,E, Kneteman,NM, Lakey,JR, Shapiro,AM: Five-year follow-up after clinical islet transplantation. *Diabetes* 54:2060-2069, 2005

2. Shapiro,AM, Ricordi,C, Hering,BJ, Auchincloss,H, Lindblad,R, Robertson,RP, Secchi,A, Brendel,MD, Berney,T, Brennan,DC, Cagliero,E, Alejandro,R, Ryan,EA, DiMercurio,B, Morel,P, Polonsky,KS, Reems,JA, Bretzel,RG, Bertuzzi,F, Froud,T, Kandaswamy,R, Sutherland,DE, Eisenbarth,G, Segal,M, Preiksaitis,J, Korbutt,GS, Barton,FB, Viviano,L, Seyfert-Margolis,V, Bluestone,J, Lakey,JR: International trial of the Edmonton protocol for islet transplantation. *N.Engl.J.Med.* 355:1318-1330, 2006

3. Korbutt,GS, Elliott,JF, Ao,Z, Smith,DK, Warnock,GL, Rajotte,RV: Large scale isolation, growth, and function of porcine neonatal islet cells. *J.Clin.Invest* 97:2119-2129, 1996

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

5. Kobayashi,T, Harb,G, Rayat,GR: 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, 2005

6. Kin,T, Korbutt,GS, Kobayashi,T, Dufour,JM, Rajotte,RV: Reversal of diabetes in pancreatectomized pigs after transplantation of neonatal porcine islets. *Diabetes* 54:1032-1039, 2005

7. Cardona,K, Korbutt,GS, Milas,Z, Lyon,J, Cano,J, Jiang,W, Bello-Laborn,H, Hacquoil,B, Strobert,E, Gangappa,S, Weber,CJ, Pearson,TC, Rajotte,RV, Larsen,CP: Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat.Med.* 12:304-306, 2006

8. Arefanian,H, Tredget,EB, Rajotte,RV, Gill,RG, Korbutt,GS, Rayat,GR: Short-Term Administrations of a Combination of Anti-LFA-1 and Anti-CD154 Monoclonal Antibodies Induces Tolerance to Neonatal Porcine Islet Xenografts. *Diabetes* 10.2337/DB09-0413: 2009

9. Rayat,GR, Korbutt,GS, Elliott,JF, Rajotte,RV: Survival and function of syngeneic rat islet grafts placed within the thymus versus under the kidney capsule. *Cell Transplant.* 6:597-602, 1997

10. Arefanian,H, Tredget,EB, Rajotte,RV, Korbutt,GS, Gill,RG, Rayat,GR: Combination of anti-CD4 with anti-LFA-1 and anti-CD154 monoclonal antibodies promotes long-term survival and function of neonatal porcine

islet xenografts in spontaneously diabetic NOD mice. *Cell Transplant.* 16:787-798, 2007

11. Gotoh,M, Maki,T, Kiyoizumi,T, Satomi,S, Monaco,AP: An improved method for isolation of mouse pancreatic islets. *Transplantation* 40:437-438, 1985

12. Scharp,DW, Kemp,CB, Knight,MJ, Ballinger,WF, Lacy,PE: The use of ficoll in the preparation of viable islets of langerhans from the rat pancreas. *Transplantation* 16:686-689, 1973

13. Wang,T, Singh,B, Warnock,GL, Rajotte,RV: Prevention of recurrence of IDDM in islet-transplanted diabetic NOD mice by adjuvant immunotherapy. *Diabetes* 41:114-117, 1992

14. Tredget,EB, Arefanian,H, Gill,RG, Rajotte,RV, Rayat,GR: Monotherapy with anti-LFA-1 monoclonal antibody promotes long-term survival of rat islet xenografts. *Cell Transplant.* 17:599-608, 2008

15. Goss,JA, Nakafusa,Y, Finke,EH, Flye,MW, Lacy,PE: Induction of tolerance to islet xenografts in a concordant rat-to-mouse model. *Diabetes* 43:16-23, 1994

16. Gordon,EJ, Woda,BA, Shultz,LD, Rossini,AA, Greiner,DL, Mordes,JP: Rat xenograft survival in mice treated with donor-specific transfusion and anti-CD154 antibody is enhanced by elimination of host CD4+ cells. *Transplantation* 71:319-327, 2001

17. Lehnert,AM, Yi,S, Burgess,JS, O'Connell,PJ: 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, 2000

18. Goss,JA, Nakafusa,Y, Finke,EH, Flye,MW, Lacy,PE: Induction of tolerance to islet xenografts in a concordant rat-to-mouse model. *Diabetes* 43:16-23, 1994

19. Gordon,EJ, Woda,BA, Shultz,LD, Rossini,AA, Greiner,DL, Mordes,JP: Rat xenograft survival in mice treated with donor-specific transfusion and anti-CD154 antibody is enhanced by elimination of host CD4+ cells. *Transplantation* 71:319-327, 2001

20. Lehnert,AM, Yi,S, Burgess,JS, O'Connell,PJ: 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, 2000

21. Rayat,GR, Gill,RG: Pancreatic islet xenotransplantation: barriers and prospects. *Curr.Diab.Rep.* 3:336-343, 2003

22. Joffre,O, Gorsse,N, Romagnoli,P, Hudrisier,D, van Meerwijk,JP: Induction of antigen-specific tolerance to bone marrow allografts with CD4+CD25+ T lymphocytes. *Blood* 103:4216-4221, 2004

23. Waldmann,H, Adams,E, Fairchild,P, Cobbold,S: Infectious tolerance and the long-term acceptance of transplanted tissue. *Immunol.Rev.* 212:301-313, 2006

24. Bemelman,F, Honey,K, Adams,E, Cobbold,S, Waldmann,H: Bone marrow transplantation induces either clonal deletion or infectious tolerance depending on the dose. *J.Immunol.* 160:2645-2648, 1998

25. Chen,ZK, Cobbold,SP, Waldmann,H, Metcalfe,S: Amplification of natural regulatory immune mechanisms for transplantation tolerance. *Transplantation* 62:1200-1206, 1996

26. Gordon,EJ, Woda,BA, Shultz,LD, Rossini,AA, Greiner,DL, Mordes,JP: Rat xenograft survival in mice treated with donor-specific transfusion and anti-CD154 antibody is enhanced by elimination of host CD4+ cells. *Transplantation* 71:319-327, 2001

27. Gordon,EJ, Woda,BA, Shultz,LD, Rossini,AA, Greiner,DL, Mordes,JP: Rat xenograft survival in mice treated with donor-specific transfusion and anti-CD154 antibody is enhanced by elimination of host CD4+ cells. *Transplantation* 71:319-327, 2001

28. Lee,I, Wang,L, Wells,AD, Dorf,ME, Ozkaynak,E, Hancock,WW: Recruitment of Foxp3+ T regulatory cells mediating allograft tolerance depends on the CCR4 chemokine receptor. *J.Exp.Med.* 201:1037-1044, 2005

29. Curiel,TJ, Coukos,G, Zou,L, Alvarez,X, Cheng,P, Mottram,P, Evdemon-Hogan,M, Conejo-Garcia,JR, Zhang,L, Burow,M, Zhu,Y, Wei,S, Kryczek,I, Daniel,B, Gordon,A, Myers,L, Lackner,A, Disis,ML, Knutson,KL, Chen,L, Zou,W: Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat.Med.* 10:942-949, 2004

CHAPTER 4

CELLULAR MECHANISM OF DOMINANT TOLERANCE TO NEONATAL PORCINE ISLET XENOGRAFTS INDUCED BY A COMBINATION OF ANTI-LFA-1 AND ANTI-CD154 MONOCLONAL ANTIBODIES IN MICE

4.1 INTRODUCTION

Neonatal porcine islets (NPI) are an attractive alternative source for clinical islet transplantation due to their capacity to reverse hyperglycemia in small (1-6) and large animal models (7;8) including pre-clinical non-human primate models (8). Additionally, isolating a large number of islets from a neonatal porcine pancreas is easy and reproducible. Isolated NPI can be maintained in culture with the potential capacity for proliferation and differentiation (1). We previously demonstrated that a combination of monoclonal antibodies (mAbs), particularly anti-LFA-1 and anti-CD154 mAbs, is highly effective in protecting NPI xenografts from rejection in non-autoimmune prone B6 mice (2;3). We also recently showed that this combined mAb therapy can induce species and tissue specific tolerance to NPI xenografts in B6 mice that is mediated by regulatory T cells (5;6). One of the major goals in the field of islet xenotransplantation is to develop safe and effective therapies that facilitate tolerance induction to NPI xenografts. Tolerizing regimens in clinical islet xenotransplantation is preferable as avoiding lymphocyte depletion may minimize the risks of malignancy and infection associated with T cell depletion therapy (9-11) and sustained low $CD4^+$ T cell counts (12;13). Additionally, optimal tolerizing regimens may require simultaneous modulation of immune cells such as $CD4^+$ and $CD8^+$ T cell subsets (14). In the current study our aims were to determine if a combination of anti-LFA-1 and anti-CD154 mAbs can induce dominant tolerance to NPI xenografts and also to determine the role of $CD4^+$ and $CD8^+$ T cell compartments in the induction and maintenance of tolerance to NPI

xenograft induced by this combined mAb therapy. Our results show that combined anti-LFA-1 and anti-CD154 mAbs therapy can induce dominant tolerance and $CD4^+$ T cells play a more important role in the maintenance of tolerance to NPI xenografts in non-autoimmune prone B6 mice.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Six to 8-week-old male immune-competent B6 (C57BL/6J, H-2^b) and immune-deficient B6 *rag-/-* (B6.129S7-$Rag1^{tm1Mom}$/J, H-2^b) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and were used as recipients. These mice were rendered diabetic by a single intraperitoneal (i.p.) injection of streptozotocin (Sigma, St Louis, MO, USA) 180 or 175 mg/kg body weight for B6 or B6 *rag-/-* mice 4-6 days before transplantation, respectively. Blood samples were obtained from the tail vein and glucose levels were monitored using a Precision glucose meter (ONETOUCH Ultra, Lifescan, Milpitas, CA, USA). All diabetic mice had two consecutive non-fasting blood glucose levels (BGLs) >20 mmol/l prior to islet transplantation. All mice were fed standard laboratory food and cared for according to the guidelines established by the Canadian Council on Animal Care committee. Three-day-old Duroc cross neonatal pigs (>1.5 kg body weight) were purchased from the University of Alberta (Edmonton, Alberta, Canada) as islet donors.

4.2.2 Islet isolation and transplantation

Neonatal porcine islets (NPI) were isolated as previously described (1). Briefly, neonatal pigs were anesthetized with halothane and subjected to

laparotomy and exsanguination. The pancreas was removed, placed in Hanks' balanced salt solution (HBSS, Sigma, St Louis, MO, USA) cut into small pieces, and digested with 2.5 mg/ml collagenase (clostridiopeptidase A, type XI, Sigma-Aldrich, St. Louis, MO, USA). Digested tissue was filtered through a 500 μm nylon screen then cultured for 7 days in HAM's F10 medium (GIBCO Laboratories, Grand Island, NY, USA) containing 10 mmol/l glucose, 50 μmol/l isobutylmethylxanthine (ICN Biomedicals, Montreal, Canada), 0.5% bovine serum albumin (fraction V, radioimmunoassay grade; Sigma), 2 mmol/l L-glutamine, 3 mmol/l $CaCl_2$, 10 mmol/l nicotinamide (BDH Biochemical, Poole, England), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C (5% CO_2, 95% air). After 7 days of culture, NPI were counted for transplantation and a total of 2,000 NPI were transplanted under the left kidney capsule of B6 or B6 *rag-/-* mice that had two consecutive non-fasting blood glucose levels of >20 mmol/l 4 to 6 days after STZ injection (1;2). Engraftment was considered successful when blood glucose level reached ≤8.5 mmol/l. Graft rejection was defined as the first of 3 consecutive days of hyperglycemia (>12 mmol/l). Rejection was confirmed by histological analysis of the graft. Nephrectomy of the graft-bearing kidney was performed on recipients with long-term graft function to confirm that normoglycemia was due to the islet xenograft.

4.2.3 Monoclonal antibody therapies

B6 mouse recipients of NPI were designated to receive short-term administration of anti-LFA-1 mAb (KBA; rat IgG2a; prepared as ascites; 200 μg on days 0, 1, 7, 14 post-transplant) and anti-CD154 mAb (MR-1; hamster IgG1;

Bio Express, West Lebanon, NH, USA; 250 μg on days –1 and 1 and 2 times a week for an additional 4 weeks post-transplant). Anti-CD4 mAb (GK1.5, rat IgG2b; BioExpress, 10mg/kg body weight on days -1, 0, 1, 2 post-transplantation) and/or anti-CD8 mAb (2.43, rat IgG2b; BioExpress,10mg/kg body weight on days -1, 0, 1, 2 post-transplantation) were administered alone or in combination with anti-LFA-1 and anti-CD154 mAbs as described above.

4.2.4 Immunohistological analysis

Insulin-producing beta cells and immune cell infiltrates were examined in sections of graft bearing kidneys by immunohistochemistry staining. Briefly, graft-bearing kidneys were harvested and fixed in 10% buffered formalin solution and embedded in paraffin, 5 μm sections were stained with guinea pig anti-porcine insulin primary antibody (1:1,000; DAKO laboratories, Mississauga, ON, Canada) for 30 min, followed by the addition of biotinylated goat anti-guinea pig IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). Avidin-biotin complex/horseradish peroxidase (ABC/HP; Vector Laboratories, Burlingame, CA, USA) and 3, 3-diaminobenzidinetetrahydrochloride (DAB; BioGenex, San Ramon, CA, USA) were used to detect positive cells (brown color). All paraffin sections were counter-stained with Harris' hematoxylin and eosin.

4.2.5 Detection of anti-porcine antibodies by flow cytometry

Humoral immune response of B6 mouse recipients of NPI was determined by measuring serum levels of mouse anti-porcine IgG antibodies using flow cytometry. Spleen cells ($1x10^6$) obtained from NPI donors were incubated with

1:128 dilutions of mouse serum for 1 h at 37°C, 5% CO_2, and 95% air. Spleen cells were then washed with PBS and incubated with 1:200 dilution of FITC-conjugated rat adsorbed goat anti-mouse IgG antibody (Southern Biotechnology Associates, Inc. Birmingham, Alabama, AL, USA) for 1 h at 4°C. The percentage of cells bound to the antibody was detected from single parameter fluorescence histograms on a BD FACS Calibur flow cytometry machine (BD Biosciences Pharmingen, Mississauga, ON, Canada) after gating on viable lymphocytes. Controls for this experiment include porcine spleen cells alone and spleen cells incubated with secondary antibody without mouse serum.

4.2.6 Adoptive transfer assay

At 150 days post-transplantation, 50 x 10^6 spleen cells or 10 x 10^6 purified $CD4^+$ or $CD8^+$ T cells from naïve non-transplanted B6 and/or tolerant B6 mouse recipients of NPI xenografts in a total volume of 200 µl PBS were injected into the peritoneum of NPI transplanted B6 *rag-/-* mice. $CD4^+$ and $CD8^+$ T cells were purified using Cellect T cell immune affinity enrichment columns according to the manufacturer's protocol (Cedarlane Laboratories, Hornby, ON, Canada). Blood glucose levels were monitored three times a week for 60 days post-cell injection. At the time of rejection or at 60 days post-cell injection, NPI xenografts were harvested and were examined for the presence of insulin positive cells and immune cells. A survival nephrectomy was performed on B6 *rag-/-* mice that maintained normoglycemia for 60 days post-cell injection and a return to diabetic state confirmed graft function. Spleen cells were subsequently examined by flow cytometry to characterize the adoptively transferred immune cells.

4.2.7 Characterization of lymphocytes by flow cytometry

Spleen cells from naïve non-transplanted B6 and tolerant B6 mouse recipients were isolated at different time points of post-treatment or at 100 days post-transplantation, respectively. Spleen cells were isolated by mechanical disruption of spleen tissue between rough edges of glass slides. Erythrocytes from spleen cell suspension were depleted using red blood cell lyses buffer. Absolute lymphocyte numbers were determined by staining the cells with Trypan blue exclusion dye, and live cells were counted using a hemocytometer. One million viable spleen cells were incubated with fluorescent conjugated antibodies (1:100 dilution, eBioscience, San Diego, CA, USA) for 30 min at 4^{o}C. Spleen cells were washed with PBS and suspended in 300 μl of FACS buffer (2% FBS in PBS). Fluorescence histograms were created using a BD FACS Calibur flow cytometry machine and were used to determine the percentage of positive cells labeled with the corresponding antibodies. Controls for this experiment include unstained spleen cells from the corresponding mice and spleen cells from non-transplanted naïve B6 mice that were either not incubated or incubated with the antibodies.

4.2.8 Statistical analysis

Statistical differences between groups in expression of cell markers and mouse anti-porcine IgG antibody levels were determined using nonparametric Mann-Whitney test. Statistical differences in graft survival among groups were analyzed using the Kaplan-Meier Log rank test. All statistical tests were performed using SPSS statistical software, version 13.0 for Windows (Chicago, IL, USA). A p value of less than 0.05 was considered to be statistically significant.

4.3 RESULTS

4.3.1 Combination of anti-LFA-1 and anti-CD154 mAbs induces dominant tolerance to NPI xenografts.

We previously showed that combination of anti-LFA-1 and anti-CD154 mAbs can induce species and tissue specific tolerance to NPI xenograft through the regulatory T cell function (5;6). Therefore, to test our hypothesis that induced NPI xenograft tolerance is dominant, we performed an adoptive transfer assay using spleen cells from tolerant B6 mice alone or in combination with spleen cells from naive B6 mice and injected them into B6 *rag-/-* mouse recipients of NPI xenografts. Similar to what we previously reported (5;6) all B6 *rag-/-* mouse recipients of NPI xenografts that received 25×10^6 or 50×10^6 spleen cells from tolerant B6 mice protected their NPI xenografts and maintained normoglycemia for over 60 days post-cell transfer (Table 4.1). However, B6 *rag-/-* mouse recipients of NPI xenografts that received 25×10^6 or 50×10^6 spleen cells from naive B6 mice rejected their NPI xenografts and became diabetic by day 18 and 15 post-cell transfer respectively (Table 4.1).

To determine whether spleen cells from tolerant B6 mice could inhibit graft rejection by spleen cells from naive B6 mice, equal numbers of spleen cells from tolerant and naive mice (25×10^6 or 50×10^6 each) were co-transferred into B6 *rag-/-* mouse recipients of NPI. All of the B6 *rag-/-* mice that received combined spleen cells from tolerant and naive mice (1/1 ratio) maintained NPI xenograft survival for over 60 days post-cell transfer (Table 4.1). Moreover, majority of B6 *rag-/-* mouse recipients of NPI that received 25×10^6 spleen cells form tolerant

mice combined with 50×10^6 spleen cells from naive B6 mice (1/2 ratio) had prolonged NPI xenograft survival for over 60 days post–cell transfer and only one mouse became diabetic at 55 days post-cell transfer (Table 4.1). Taken together, these results show that combination of anti-LFA-1 and anti-CD154 mAbs can induce dominant tolerance to NPI xenografts in B6 mice.

Table 4.1: Graft survival of NPI xenografts in B6 *rag -/-* mice injected with spleen cells from either tolerant B6 and/or naive non-transplanted B6 mice.

Source of Spleen Cells	n	Graft Survival (Days post-cell transfer)
50×10^6 tolerant B6	5	>60(x5)
25×10^6 tolerant B6	5	>60(x5)
50×10^6 naive B6	5	11, 13, 15(x3) *
25×10^6 naive B6	5	13(x2), 15(x2), 18 *
50×10^6 tolerant + 50×10^6 naive B6	5	>60(x5)
25×10^6 tolerant + 25×10^6 naive B6	5	>60(x5)
25×10^6 tolerant + 50×10^6 naive B6	5	55, >60(x4)

* $p<0.003$ vs. other groups

4.3.2 $CD4^+$ T cells from tolerant B6 mice play a major role in transferring dominant tolerance to NPI xenografts induced by a combination of anti-LFA-1 and anti-CD154 mAbs.

To determine which subset of T cells ($CD4^+$ or $CD8^+$ T cells) can transfer tolerance to NPI xenografts, 10×10^6 purified $CD4^+$ and $CD8^+$ T cells (Purity >98%)from either tolerant and/or naive B6 mice were injected into the B6 *rag-/-*

mouse recipients of NPI. NPI xenograft survival of these mice was monitored by measuring blood glucose levels for over 60 days post-cell transfer. All of the B6 *rag-/-* mouse recipients of NPI received 10×10^6 purified $CD4^+$ or $CD8^+$ T cells from tolerant B6 mice maintained NPI xenograft survival for 60 days post-cell-transfer (Table 4.2). However, similar to what we reported previously (15) only B6 *rag-/-* mouse recipients of NPI that received purified $CD4^+$ but not $CD8^+$ T cells from naive B6 mice rejected their NPI xenografts by 22 days post-cell transfer (Table 4.2). To examine the capacity of $CD4^+$ or $CD8^+$ T cells in transferring dominant tolerance, purified $CD4^+$ or $CD8^+$ T cells from tolerant B6 mice in combination with 10×10^6 purified $CD4^+$ T cells or 50×10^6 unpurified spleen cells from naive B6 mice were injected into the B6 *rag-/-* mouse recipients of NPI and islet graft survival were monitored for 60 days post-cell transfer. Only purified $CD4^+$ but not $CD8^+$ T cells from tolerant mice could inhibit the NPI xenograft rejection mediated by purified $CD4^+$ T cells from naïve B6 mice (Table 4.2). Both purified $CD4^+$ and $CD8^+$ T cells from tolerant mice could significantly ($p< 0.003$) suppress xenoreactivity of the spleen cells from naïve B6 mice that were injected into the B6 *rag-/-* mouse recipients of NPI (Table 4.2). A statistically significant difference ($p<0.004$) was determined between B6 *rag-/-* mice that received a combination of unpurified spleen cells from naive B6 mice with purified $CD4^+$ or $CD8^+$ T cells from tolerant B6 mice (Table 4.2). Collectively, these results indicate that tolerant $CD4^+$ T cells play the major role in the transferring dominant tolerance to NPI xenografts induced by a combination of anti-LFA-1 and anti-CD154 mAbs. However tolerant $CD8^+$ T

cells also are capable to inhibit NPI xenograft rejection mediated by naive B6 spleen cells.

Table 4.2: Graft survival of NPI xenografts in B6 *rag* -/- mice injected with purified $CD4^+$ and/or $CD8^+$ T cells from either tolerant B6 or naive non-transplanted B6 mice.

Type of Transferred Cells	No. of Cells	n	Graft Survival (Days post-cell transfer)
Tolerant $CD4^+$ T cells	10×10^6	5	>60(x5) [a]
Tolerant $CD8^+$ T cells	10×10^6	5	>60(x5) [b]
Naïve $CD4^+$ T cells	10×10^6	5	13, 15, 18, 22(x2) *
Naïve $CD8^+$ T cells	10×10^6	5	>60(x5) [c]
Tolerant $CD4^+$ T cells + Naïve $CD4^+$ T cells	10×10^6 (Each type)	5	>60(x5) [d]
Tolerant $CD8^+$ T cells + Naive $CD4^+$ T cells	10×10^6 (Each type)	5	10, 14, 17, 20 (x2) *
tolerant $CD4^+$ T cells + naïve spleen cells	10 and 50 $\times10^6$ (Respectively)	5	51, 56, >60(x3) [e]
Tolerant $CD8^+$ T cells + Naïve spleen cells	10 and 50 $\times10^6$ (Respectively)	5	24, 31, 42, 48, 51†
Naive spleen cells	50×10^6	5	11, 13, 15(x3) [f] *

* $p<0.004$ vs. groups **a, b, c, d**, and **e**
† $p<0.003$ vs. groups **a, b, d, e**, and **f**

4.3.3 Induction and maintenance of tolerance to NPI xenografts induced by a combination of anti-LFA-1 and anti-CD154 mAbs does not require $CD4^+$ or $CD8^+$ T cells.

We explored the pattern of depletion and recurrence of new population of $CD4^+$ and $CD8^+$ T cells by using short-term administrations of depleting anti-CD4 and anti-CD8 mAbs in naive non-transplanted B6 mice. Spleen cells from these mice were isolated and were analyzed for the presence of $CD4^+$ and $CD8^+$ T cells at different time points of post-mAb administration (n=3 in each time point per each group) using flow cytometry. The percentage of $CD4^+$ and $CD8^+$ T cells in naive B6 were reduced to <% 1.5 on day 6 post-injection of anti-CD4 and/or anti-CD8 mAbs. However on day 56 post-injection new populations of $CD4^+$ and/or $CD8^+$ T cells were detected and on day 66 post-injection all of the mAb treated B6 mice had a comparable amount of $CD4^+$ and/or $CD8^+$ T cells with those detected in untreated B6 mice (Figure 4.1A and B, respectively). To determine the role of $CD4^+$ and/or $CD8^+$ T cells in the induction of tolerance to NPI xenografts using short-term administration of a combination of anti-LFA-1 and anti-CD154 mAbs, B6 mouse recipients of NPI were simultaneously treated with a combination anti-LFA-1 and anti-CD154 mAbs plus anti-CD4 and/or anti-CD8 mAbs. All B6 mouse recipients of NPI which were simultaneously treated with a combination of anti-LFA-1 and anti-CD154 mAbs alone or plus anti-CD4 and/or anti-CD8 mAbs achieved normoglycemia and maintained NPI xenograft survival for >100 days post-transplnatation (Table 4.3).

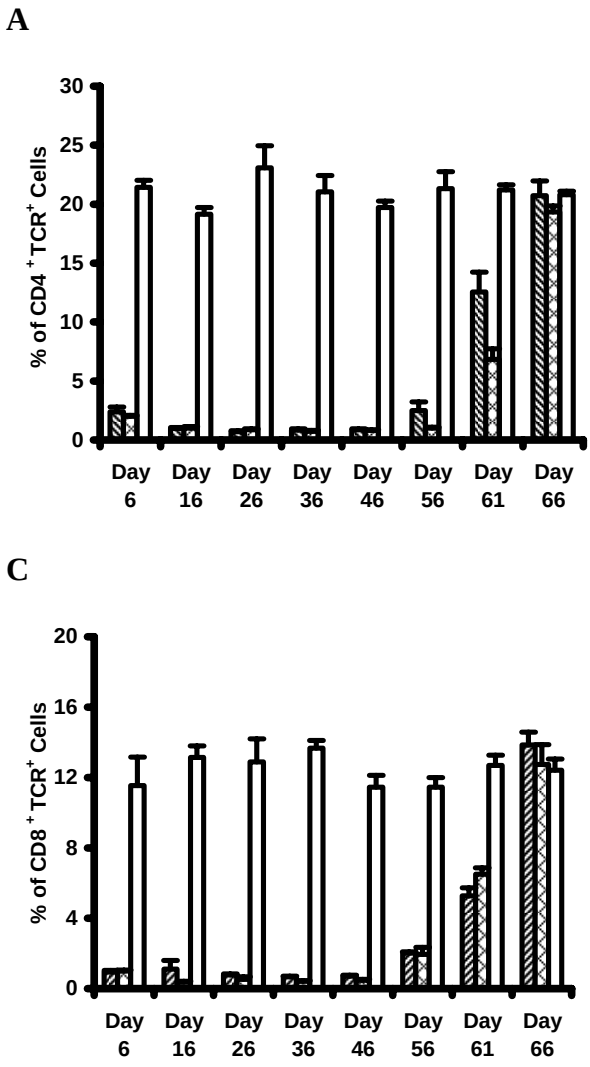

Figure 4.1: Frequency of $CD4^+$ and $CD8^+$ T cells in the spleen cells from naive non-transplanted B6 mice treated with short-term administrations of anti-CD4 and/or anti-CD8 mAbs. The percentage of both $CD4^+$ and $CD8^+$ T cells in the treated naive B6 micewere reduced to <% 1.5 on day 6 post injection of anti-CD4 mAb (black downward diagonal, 10mg/kg body weight on days 0, 1, 2, and 3 post-injection), anti-CD8 mAb (black upward diagonal, 10mg/kg body weight on days 0, 1, 2, and 3 post-injection), or both (outlined diamond). However, on day 56 post injection new populations of $CD4^+$ and/or $CD8^+$ T cells were detected on day 66 post injection all of the treated non-transplanted B6 mice had a comparable amount of $CD4^+$ and/or $CD8^+$ T cells with untreated naive B6 mice (A and B, respectively).

Table 4.3: Long-term survival of NPI xenografts in B6 mouse recipients of NPI treated with short-term administrations of anti-LFA-1 and anti-CD154 mAbs alone or plus anti-CD4 and/or anti-CD8 mAbs.

Group	n	Graft Survival (Days post-transplantation)
Anti-LFA-1 + anti-CD154 mAbs	7	>100 (x7)
Anti-LFA-1 + anti-CD154 + anti-CD4 mAbs	8	>100 (x8)
Anti-LFA-1 + anti-CD154 + anti-CD8 mAbs	8	>100 (x8)
Anti-LFA-1 + anti-CD154 + anti-CD4 + anti-CD8 mAbs	8	>100 (x8)

Histological analysis of the NPI xenografts harvested from mice that were treated with a combination of anti-LFA-1 and anti-CD154 mAbs alone or plus anti-CD4 and/or anti-CD8 mAbs showed intact islets with insulin producing cells and no immune cell infiltration (Figure 4.2A-D, respectively). hese characterization were comparable to what was seen in the NPI xenografts harvested from immune-deficient B6 *rag-/-* mice transplanted with same NPI (Figure 4.2E). In addition, the amount of anti-porcine antibody production of B6 mouse recipients of NPI treated with a combination of anti-LFA-1 and anti-CD154 mAbs alone or plus anti-CD4 and/or anti-CD8 mAbs to NPI xenografts (Figure 4.2F-I, respectively) were comparable to the amount detected in naive non-transplanted B6 mice (Figure 4.2J).

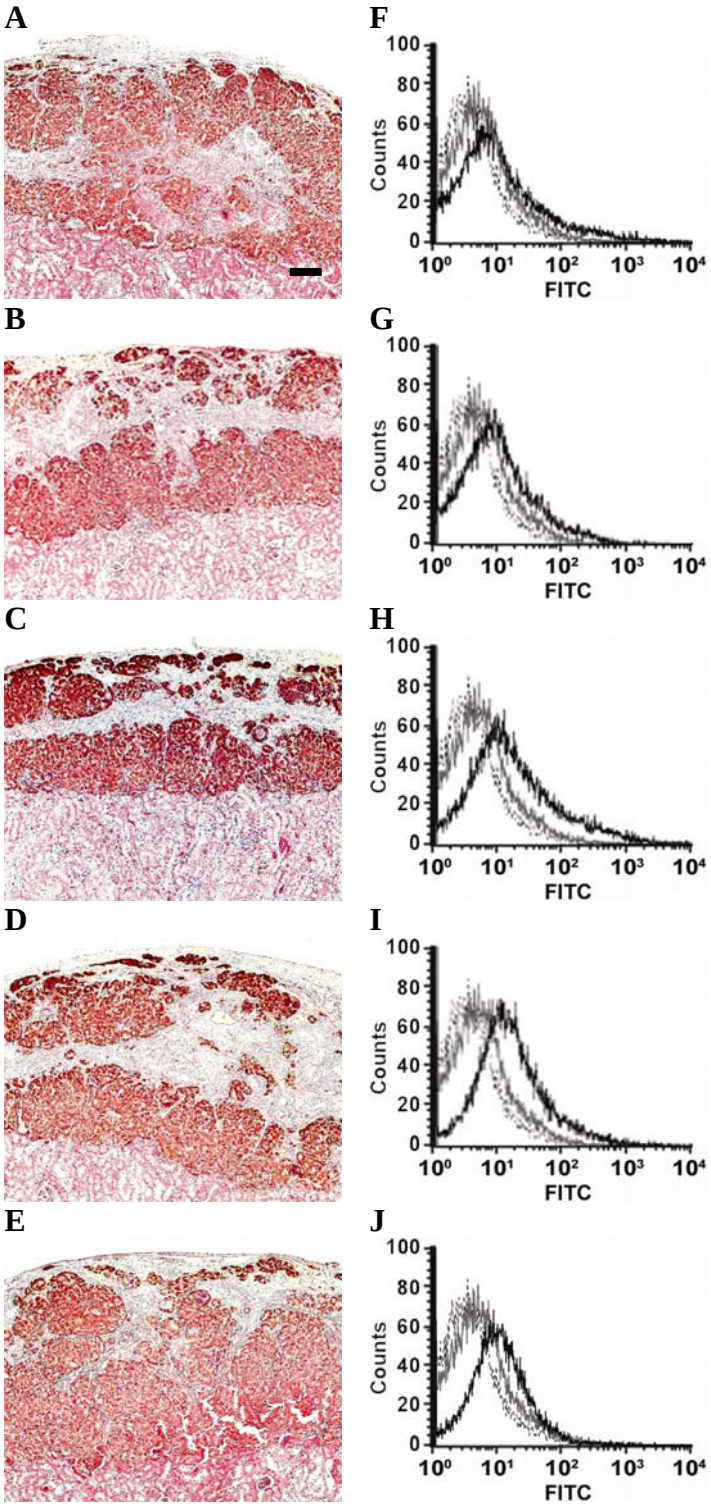

Figure 4.2: Representative NPI xenograft and anti-porcine Ab levels from B6 mice treated with a combination of anti-LFA-1 and anti-CD154 mAbs alone or plus anti-CD4 and/or anti-CD8 mAbs. ***A-E:*** NPI xenografts in B6 mouse recipients treated with a combination of anti-LFA-1 and anti-CD154 mAbs alone (A) or plus anti-CD4 mAb (B) or anti-CD8 mAb (C) or combination of anti-CD4

and anti-CD8 mAbs (D) remained intact with abundant insulin-positive cells comparable to the NPI xenografts in immune-deficient B6 *rag-/-* mice(E). Scale bar represents 100 μm. ***H-J:*** The levels of IgG antibodies from tolerant B6 mouse recipients of NPI treated with a combination of anti-LFA-1 and anti-CD154 mAbs alone (5.3%-7.6%, n=7, A) or plus anti-CD4 mAb (5.9 % - 7.8%, n=8, B) or anti-CD8 mAb (4.9% -6.9%, n=8, C) or anti-CD4 and anti-CD8 mAbs (7.9%-8.6%, n=8, D) were comparable to the amount of anti-porcine IgG antibodies detected in the serum of naive non-transplanted B6 (4.9%-7.3%, n=5, D). Controls for this experiment consisted of unstained (dashed black line) and secondary antibody without serum (solid gray line).

Further characterization of the spleen cells isolated from all of the treated groups showed comparable amount of T and B cells (Figure 4.3A). However significantly higher expression of regulatory markers namely CD25, foxp3 and GITR, and co-inhibitory markers such as PD-1 and CTLA-4 were detected in these mice (Figure 4.3B-D, respectively) compared to non-transplanted naive B6 mice. Based on these results, induction of tolerance to NPI xenografts by a combination of anti-LFA-1 and anti-CD154 mAbs does not require the presence of CD4 or CD8 T cells.

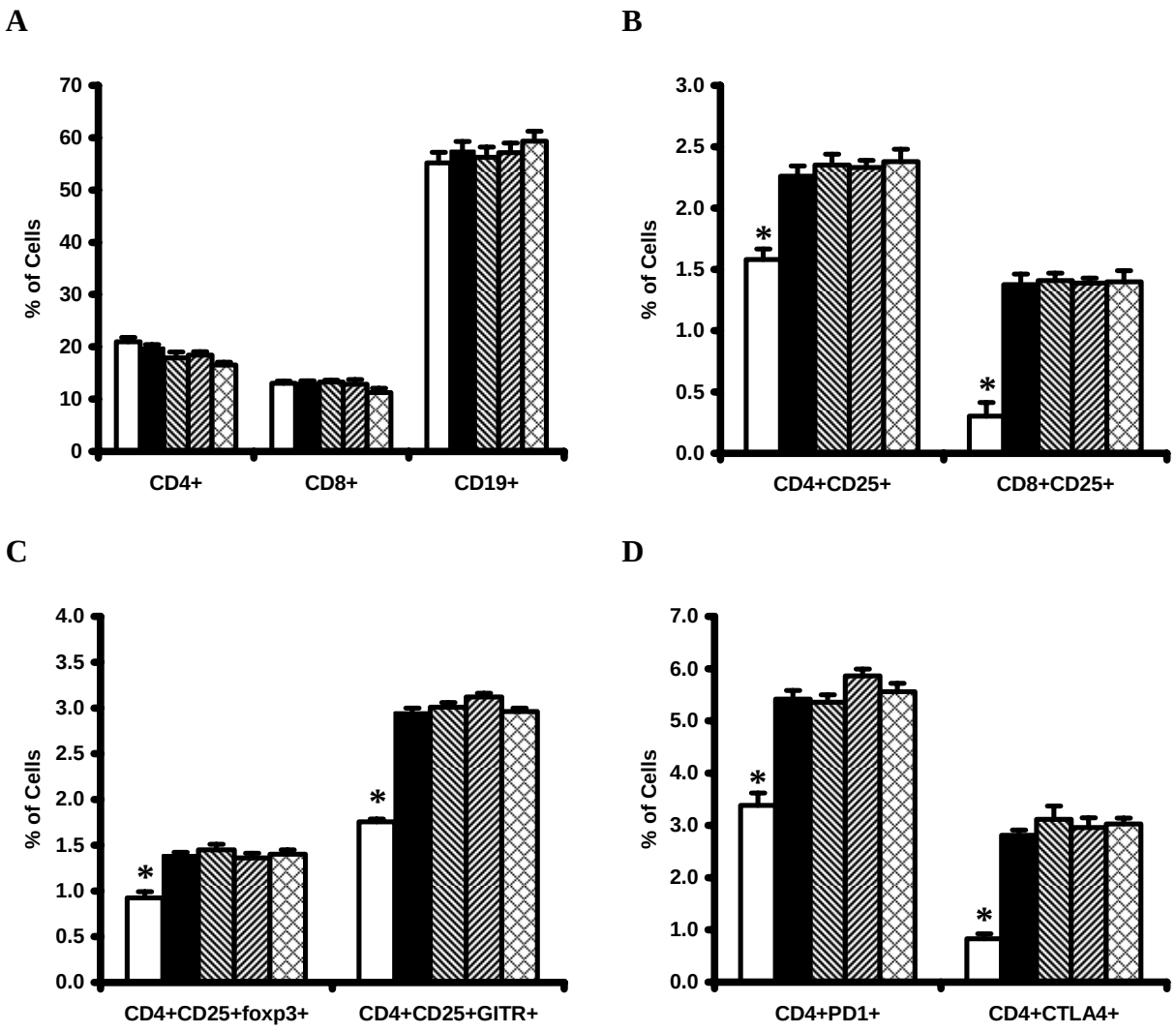

Figure 4.3: Characterization of the spleen cells isolated from B6 mouse recipients of NPI treated with a combination of anti-LFA-1 and anti-CD154 mAbs alone or plus anti-CD4 and/or anti-CD8 mAbs. Frequency of $CD4^+$, $CD8^+$ T and $CD19^+$ B cells isolated from the spleen of B6 mouse recipients of NPI treated with a combination of anti-LFA-1 and anti-CD154 mAbs alone (black bars) or plus anti-CD4 (black downward diagonal) or plus anti-CD8 (black upward diagonal) or both (outlined diamond) were comparable with the percentages of these cells isolated from the spleen of naive-nontransplanted B6 mice (A). The frequency of $CD4^+CD25^+$and $CD8^+CD25^+$, $CD4^+CD25^+foxp3^+$ and $CD4^+CD25^+GITR^+$ cells expressing regulatory and $CD4^+PD1^+$ and $CD4^+CTLA4^+$ cells expressing co inhibitory markers were significantly higher ($p<0.001$) in all different groups of mAbs treated B6 mouse recipients of NPI xenografts compared to those found in naive non-transplanted B6 mice (B-D).

While tolerance to NPI xenografts induced by a combination of anti-LFA-1 and anti-CD154 mAbs does not require $CD4^+$ and/or $CD8^+$ T cells we further examined the role of $CD4^+$ and $CD8^+$ T cells in the maintenance of tolerance by treating some of tolerant B6 mice with anti-CD4 and/or anti-CD8 mAbs. The majority (3 out of 5) of the tolerant B6 mice treated with anti-CD4 mAb maintained NPI xenograft survival for >100 days post-treatment (>250 days post-transplantation) while two mice rejected their NPI xenografts on days 76 and 81 post-treatment (226 and 231 days post-transplantation, respectively, Table 4.4). In contrast all of the tolerant mice treated with anti-CD8 mAb maintained NPI xenograft survival for >100 days post-treatment (>250 days post-transplantation, Table 4.4). All tolerant mice treated with a combination of anti-CD4 and anti-CD8 mAbs rejected their NPI xenografts and became diabetic by day 88 post-treatment (238 days post-transplantation, $p=0.062$ vs. anti-CD4 treated group and $p<0.001$ vs. untreated and anti-CD8 treated groups, Table 4.4). Taken together this results indicate that $CD4^+$ and/or $CD8^+$ T cells play an important role in the maintenance of tolerance to NPI xenografts induced by a combination of anti-LFA-1 and anti-CD154 mAbs.

Table 4.4: NPI xenograft survival in tolerant B6 mice treated with short-term administration of anti-CD4 and/or anti-CD8 mAbs.

Group	n	Graft Survival (Days post-transplantation)
Untreated	5	>250 (x5)
anti-CD4 mAb treated	5	226, 231, >250 (x3)
anti-CD8 mAb treated	5	>250 (x5)*
anti-CD4 + anti-CD8 mAbs treated	5	215, 220, 226, 236, 238 †‡§

* p=0.134 vs. untreated mice or treated mice with anti-CD4 mAb
† p=0.062 vs. treated mice with anti-CD4 mAb
‡ p<0.002 vs. treated mice with anti-CD8 mAb
§ p<0.001 vs. untreated mice

4.3.4 Immune modulation of antigen presenting cells by a combination of anti-LFA-1 and anti-CD154 mAbs can delay NPI xenograft rejection by naïve $CD4^+$ T cells.

We determined whether immune cells from tolerant B6 mice were remained bound by anti-LFA-1 (KBA, rat IgG2a) and anti-CD154 (MR-1; hamster IgG1) mAbs at day 150 post-transplantation using flow cytometry. Incubation of spleen cells from tolerant B6 mice with biotinylated anti-rat IgG and anti-hamster IgG mAbs resulted in undetectable binding (Figure 4.5A and B, respectively) meaning lack of the presence of anti-LFA-1 (KBA) and anti-CD154 (MR-1) mAbs bound to the cells. However, incubation of those cells with anti-LFA-1 (KBA) and anti-CD154 (MR-1) mAbs prior to incubation with corresponding secondary antibodies (biotinylated anti-rat IgG and anti-hamster

IgG mAbs, respectively) significantly higher percentages of cells bound by mAbs were detected (Figure 4.4A and B, n=5, $p<0.001$, respectively). This pattern was comparable to the binding of the secondary antibodies to anti-LFA-1 and anti-CD154 mAbs on spleen cells from naïve non-transplanted B6 mice (Figure 4.4C and D).

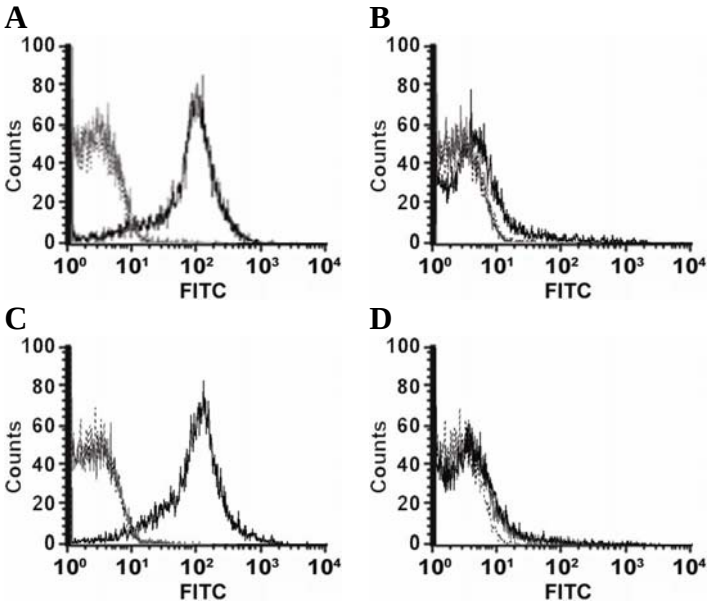

Figure 4.4: Expression of LFA-1 and CD154 molecules on the spleen cells of tolerant and naive non-transplanted B6 mice. Incubation of spleen cells from tolerant B6 mice with biotinylated anti-rat IgG and anti-hamster IgG mAbs resulted in undetectable binding of anti-LFA-1 and anti-CD154 mAbs (solid gray line, 1.13%±0.07; A and 0.61%±0.07; B, n=5, respectively). However, when spleen cells from tolerant B6 mice were first incubated with anti-LFA-1 and anti-CD154 mAbs prior to incubation with biotinylated secondary antibodies; significantly higher percentages of spleen cells ($p<0.001$) were detected bound by anti-LFA-1 and anti-CD154 mAbs (solid black line, 83.39%±1.50, A and 4.85%±1.07, B, n=5, respectively). This binding was comparable to the binding of anti-LFA-1 and anti-CD154 mAbs detected on spleen cells from naïve non-transplanted B6 mice (solid black line, 82.37%±1.76, C and 3.76%±0.82, D, respectively).

The percentage of spleen cells from tolerant and naïve non-transplanted B6 mice that were bound to anti-LFA-1 (KBA) were 83.39%±1.50 and 82.37%±1.76 and for anti-CD154 (MR-1) were 4.85±1.07 and 3.76±0.82, respectively (n=5 in each group, Table 4.5). We were also interested to further characterize the phenotype of immune cells bound by anti-LFA-1 and anti-CD154 mAbs using flow cytometry. We gated on LFA-1^+ and $CD154^+$ cells and we found that $CD4^+$ and $CD8^+$ T cells composed only a small portion of immune cells which bound by anti-LFA-1 and anti-CD154 mAbs (Table 4.5). In addition to T cells, $CD19^+$ B cells, $CD11c^+$ dendritic cells (DC), $F4/80^+$ macrophages and $NK1.1^+$ NK cells were detected to be bound by anti-LFA-1 and anti-CD154 mAbs (Table 4.5). Moreover, significantly lower percentage of $CD11c^+CD154^+$ and $CD11c^+$LFA-1^+ cells were detected from tolerant B6 mouse compared to the same population from naive non-transplanted B6 mouse spleen cells ($p<0.016$, n=5, Table 4.5). In contrast significantly higher percentage of $F4/80^+CD154^+$ and $F4/80^+$LFA-1^+ were detected in spleen cells from tolerant B6 mice compared to the naive non-transplanted B6 mouse spleen cells ($p<0.03$, n=5 in each group, Table 4.5).

To determine the effect of a combination of anti-LFA-1 and anti-CD154 mAbs on antigen presenting cells (APC) particularly, DC and macrophages, immune deficient B6 *rag-/-* mice which has not functional T and B cells were transplanted with NPI and treated with a combined mAbs therapy.

Table 4.5: Characterization of the phenotype of immune cells from tolerant B6 and naïve non-transplanted B6 mice bound by anti-LFA-1 and anti-CD154 mAbs.

	% of Total LFA-1$^+$ Cells	Gated on LFA-1$^+$ Cells					
		% CD4$^+$	% CD8$^+$	% CD19$^+$	% CD11c$^+$	% NK1.1$^+$	% F4-80$^+$
Tolerant B6 mice	83.39±1.50	22.54±0.90	13.86±0.25	59.53±1.68	2.93±0.45*	0.19±0.11	6.77±0.30†
Naive B6 mice	82.37±1.76	21.94±0.49	13.34±0.70	57.49±0.89	4.88±0.39	0.08±0.02	5.10±0.45
	% of Total CD154$^+$ Cells	Gated on CD154$^+$ Cells					
		% CD4$^+$	% CD8$^+$	% CD19$^+$	% CD11c$^+$	% NK1.1$^+$	% F4-80$^+$
Tolerant B6 mice	4.85±1.07	10.30±0.40	2.73±0.15	62.93±1.25	17.45±0.52*	0.85±0.09	10.27±0.96†
Naive B6 mice	3.76±0.82	9.46±0.33	2.30±0.10	60.66±1.67	23.32±1.57	1.06±0.04	6.43±0.63

* $p<0.016$ vs. Naive B6
† $p<0.03$ vs. Naive B6
n=5 in each group

The levels of anti-LFA-1 and anti-CD154 mAbs in the serum of these mice were detected in different time points using flow cytometry. At 60 days post-transplantation when the level of mAbs in the serum of the B6 *rag-/-* mouse recipients of NPI treated with a combination of anti-LFA-1 and anti-CD154 mAbs were undetectable the mice were injected with 10×10^6 purified $CD4^+$ T cells from naive non-transplanted B6 mouse spleen cells and NPI xenograft survival were monitored for 60 days post-cell transfer. Majority (3 out of 5) of reconstituted B6 *rag-/-* mouse recipients of NPI xenografts achieved normoglycemia and one mouse maintained normoglycemia for over 60 days post-cell transfer. The other two mice became diabetic on days 46 and 53 post-cell transfer (Table 4.6). However all of the B6 *rag-/-* mouse recipients of NPI which were not treated with a combination of anti-LFA-1 and anti-CD154 mAbs and received the same cell population from naive non-transplanted B6 mice did not achieve normoglycemia indicating NPI xenograft rejection ($p<0.05$, Table 4.6). Taken together, these data show that a combination of anti-LFA-1 and anti-CD154 mAbs could modulate the other parts of immune cell compartments except T and B cells such as APC. This immune modulation of APC could result in the delay of NPI xenograft rejection mediated by $CD4^+$ T cells.

Table 4.6: Graft survival of NPI xenografts in B6 *rag -/-* mice treated with combination of anti-LFA-1 and anti-CD154 mAbs injected with 10×10^6 purified $CD4^+$ T cells from naive non-transplanted B6 mice.

Group	n	Graft Survival (days post-cell transfer)	No. of Mice Achieved Normoglycemia
Untreated	5	0(x5)	0
anti-CD154 + anti-LFA-1	5	0(x2), 46, 53, >60 *	3

* $p<0.05$ vs. untreated group

4.4 DISCUSSION

We recently showed that short-term administrations of a combination of anti-LFA-1 and anti-CD154 mAb therapy resulted in species and tissue specific tolerance to NPI xenografts that is mediated by T regulatory cells (5;6). In the current study, we demonstrated that tolerance induced by this combined mAb therapy is dominant and $CD4^+$ T cells play a more important role than $CD8^+$ T cells in transferring tolerance to NPI xenografts; a phenomena also shown using this mAb combination in an allogeneic model of islet transplantation (16). Similar to what we showed previously (5;6), all immune deficient B6 *rag-/-* mouse recipients of first or second party NPI xenografts reconstituted with spleen cells from tolerized mice maintained normoglycemia for >60 days post-cell transfer suggesting that immune cells in treated B6 mice do not have the capacity to reject the NPI xenografts. We also found that lymphocytes from tolerant mice are capable of suppressing the xenoreactivity of lymphocytes from naïve non-transplnted B6 mice in a dose-dependent manner (1/1 and 1/2 ratio). The total number of combined spleen cells from tolerant and naive B6 mice reconstituted in

B6 *rag-/-* mice did not significantly alter the rejection rate (in concentrations of 100×10^6 cells/mouse or 50×10^6 cells/mouse). No significant difference was detected between the groups of B6 *rag-/-* mice that received either a combination of spleen cells from each tolerant and naive B6 mice of 50×10^6 (total 100×10^6) or 25×10^6 (total 50×10^6), respectively.

In an effort to delineate the specific T cell subsets responsible for tolerance we showed in the adoptive experiment that $CD4^+$ T cells from tolerant B6 mice did not reject the established NPI xenografts healed in B6 *rag-/-* mice. We also found similar to what were reported by our group and others that naive $CD4^+$ T cells but not $CD8^+$ T cells play major role in islet xenograft rejection (17-19). Our results further demonstrate that purified $CD4^+$ T cells from tolerant mice can suppress the xenoreactive function of both purified $CD4^+$ T cells and unpurified spleen cells derived from naive B6 mice. This phenomenon could be due to a higher percentage of $CD4^+$ T regulatory cells in tolerant spleen cells which we have previously demonstrated to have higher expression of CD25, foxp3, GITR regulatory and co-inhibitory markers, PD1 and CTLA4 (5). Interestingly we found that tolerant $CD8^+$ T cells can also significantly delay NPI xenograft rejection when combined with unpurified spleen cells but not in combination with purified $CD4^+$ T cells from naive non-transplanted B6 mice. A possible mechanism for this phenomena could be due to the regulatory phenotype and function of $CD8^+$ T cells from tolerant B6 mice (20) as we found a significantly higher percentage of $CD8^+CD25^+$ T cells in tolerant B6 mice compared to naïve non-transplanted B6 mice (5).

While $CD4^+$ and $CD8^+$ T cells may have tolerizing properties in treated mice, our results suggest that they are involved in the maintenance of tolerance to NPI xenografts induced by a combination of anti-LFA-1 and anti-CD154 mAbs. We showed an acute depletion and reoccurrence of new population of $CD4^+$ and $CD8^+$ T cells after using depleting anti-CD4 and anti-CD8 mAbs on day 6 and 56 days post-treatment, respectively which was also reported by others (21;22). Depletion of $CD4^+$ or $CD8^+$ T cells from tolerant mice was not significantly effective in breaking tolerance to NPI xenografts. However depletion of both $CD4^+$ and $CD8^+$ T cells from tolerant mice resulted in the rejection of NPI xenografts ($p<0.001$) which was significant compared to the tolerant mice which did not received any depleting mAbs treatment. These data suggest that tolerant T cell subsets may compensate for the effect of each other in the maintenance of tolerance to NPI xenografts.

In order to explore the role of $CD4^+$ and $CD8^+$ T cells in induction of tolerance to NPI xenografts by a combination of anti-LFA-1 and anti-CD154 mAbs we treated B6 mouse recipients of NPI simultaneously with anti-LFA-1 and anti-CD154 mAbs plus anti-CD4 and/or anti-CD8 mAbs. No significant differences were detected in regards of T and B cell compartments, T cell subsets expressing regulatory and co inhibitory markers, amount of anti-porcine antibody production, and presence of insulin positive cells in the grafts site between the group of combined anti-LFA-1 and anti-CD154 mAb treated mice with or without anti-CD4 and/or anti-CD8 mAbs. These data suggest that $CD4^+$ and/or $CD8^+$ T cells may not be required for induction of tolerance to NPI xenografts induced by

a combination of anti-LFA-1 and anti-CD154 mAbs. An indefinite NPI xenogaft survival and comparable T and B cell phenotypes and function amongst of all groups suggests that some other population of immune cells except T cell subsets could be targeted by the combination of anti-LFA-1 and ant-CD154 mAbs and those cells may support induction of tolerance to NPI xenograft by expanding of regulatory T cell subsets.

Accessibility of the ligands for anti-LFA-1 and anti-CD154 mAbs on B cells, dendritic cells, macrophages, and NK cells from either tolerant or naive non-transplanted B6 mice could provide another evidence for the role of other components of immune cells besides T cell subsets in the induction of tolerance to NPI xenografts by a combination of anti-LFA-1 and anti-CD154 mAbs. Significantly lower percentages of $CD11c^+$ $LFA\text{-}1^+$ and $CD11c^+$ $CD154^+$ DCs and higher percentages of $F4/80^+$ $LFA\text{-}1^+$ and $F4/80^+$ $CD154^+$ from tolerant spleen cells in comparison to the same population of spleen cells from naive B6 mice could be considered as a possible phenotype for higher percentage of tolerogenic APC which could play a role in the induction of peripheral tolerance (23-30) and could induce an inhibitory feedback loop with T regulatory cells in the induced tolerance (31). However in our case it needs to be investigated further.

To determine the possible effect of a combination of anti-LFA-1 and anti-CD154 mAbs on APC *in vivo*, B6 *rag-/-* mouse recipients of NPI were treated with the tolerogenic combination of mAbs. The level of anti-LFA-1 (KBA) and anti-CD154 (MR-1) mAbs in the sera of the treated B6 *rag-/-* mouse recipients of NPI was undetectable on day 60 post-transplantation. Anti-CD154 mAb (MR-1)

has previously been demonstrated to have a half-life of 12 days in mouse serum (32;33). Anti-LFA-1 mAb (M17/4.4.11.9, rat IgG2a isotype) demonstrated a half-life of 19 days in host serum and was undetectable at 60 days following transplantation (34). However analysis of anti-LFA-1 mAb (KBA, rat IgG2a isotype) in serum after treatment, to our knowledge, has not previously been reported. A significant improvement in the NPI xenograft survival was detected between the group of B6 *rag-/-* mouse recipients of NPI treated with a combination of anti-LFA-1 and anti-CD154 mAbs injected with purified naive $CD4^+$ T cells with the group of untreated B6 *rag-/-* mouse recipients of same NPI injected with the same purified $CD4^+$ T cells from naïve B6 mice. However a significant difference was detected in the NPI xenograft survival in the group of treated B6 *rag-/-* mice with mAbs injected with $CD4^+$ T cells from naive B6 mice compared with the group of B6 mouse recipients of NPI treated with a combination of anti-LFA-1 and anti-CD154 mAb plus anti-CD4 and anti-CD8 mAbs which could be due to the interaction of even very low amount of $CD4^+$ or $CD8^+$ T cells (<%1.5) in the treated B6 mice with the other immune cell compartments such as DC and macrophages. Taken together this data show that a combination of anti-LFA-1 and anti-CD154 mAbs can induce dominant tolerance to NPI xenografts by targeting T cell sub populations as well as APC which to by a T cell-APS loop of interaction tolerance can be induced and maintained and this strongly suggest that this strategy may be useful in clinical islet xenotransplnatation.

4.5 REFERENCES

1. Korbutt,GS, Elliott,JF, Ao,Z, Smith,DK, Warnock,GL, Rajotte,RV: Large scale isolation, growth, and function of porcine neonatal islet cells. *J.Clin.Invest* 97:2119-2129, 1996

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

3. Kobayashi,T, Harb,G, Rayat,GR: 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, 2005

4. Arefanian,H, Tredget,EB, Rajotte,RV, Korbutt,GS, Gill,RG, Rayat,GR: Combination of anti-CD4 with anti-LFA-1 and anti-CD154 monoclonal antibodies promotes long-term survival and function of neonatal porcine islet xenografts in spontaneously diabetic NOD mice. *Cell Transplant.* 16:787-798, 2007

5. Arefanian,H, Tredget,EB, Rajotte,RV, Gill,RG, Korbutt,GS, Rayat,GR: Short-Term Administrations of a Combination of Anti-LFA-1 and Anti-CD154 Monoclonal Antibodies Induces Tolerance to Neonatal Porcine Islet Xenografts. *Diabetes* 10.2337/DB09-0413: 2009

6. Arefanian,H, Tredget,EB, Mok,M, Rajotte,RV, Gill,RG, Korbutt,GS, Rayat,GR: Species and Tissue-Specific Tolerance to Neonatal Porcine Islet Xenografts Induced by A Combination of Anti-LFA-1 and Anti-CD154 Monoclonal Antibodies in C57BL/6J Mice. *Diabetes* 10.2337/DB09-0898: 2009

7. Kin,T, Korbutt,GS, Kobayashi,T, Dufour,JM, Rajotte,RV: Reversal of diabetes in pancreatectomized pigs after transplantation of neonatal porcine islets. *Diabetes* 54:1032-1039, 2005

8. Cardona,K, Korbutt,GS, Milas,Z, Lyon,J, Cano,J, Jiang,W, Bello-Laborn,H, Hacquoil,B, Strobert,E, Gangappa,S, Weber,CJ, Pearson,TC, Rajotte,RV, Larsen,CP: Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat.Med.* 12:304-306, 2006

9. Oh,CS, Stratta,RJ, Fox,BC, Sollinger,HW, Belzer,FO, Maki,DG: Increased infections associated with the use of OKT3 for treatment of steroid-resistant rejection in renal transplantation. *Transplantation* 45:68-73, 1988

10. Curtis,RE, Travis,LB, Rowlings,PA, Socie,G, Kingma,DW, Banks,PM, Jaffe,ES, Sale,GE, Horowitz,MM, Witherspoon,RP, Shriner,DA, Weisdorf,DJ, Kolb,HJ, Sullivan,KM, Sobocinski,KA, Gale,RP, Hoover,RN, Fraumeni,JF, Jr., Deeg,HJ: Risk of lymphoproliferative disorders after bone

marrow transplantation: a multi-institutional study. *Blood* 94:2208-2216, 1999

11. Jamil,B, Nicholls,KM, Becker,GJ, Walker,RG: Influence of anti-rejection therapy on the timing of cytomegalovirus disease and other infections in renal transplant recipients. *Clin.Transplant.* 14:14-18, 2000

12. Storek,J, Gooley,T, Witherspoon,RP, Sullivan,KM, Storb,R: Infectious morbidity in long-term survivors of allogeneic marrow transplantation is associated with low CD4 T cell counts. *Am.J.Hematol.* 54:131-138, 1997

13. Mansharamani,NG, Balachandran,D, Vernovsky,I, Garland,R, Koziel,H: Peripheral blood CD4 + T-lymphocyte counts during Pneumocystis carinii pneumonia in immunocompromised patients without HIV infection. *Chest* 118:712-720, 2000

14. Qin,S, Cobbold,SP, Pope,H, Elliott,J, Kioussis,D, Davies,J, Waldmann,H: "Infectious" transplantation tolerance. *Science* 259:974-977, 1993

15. Rayat,GR, Johnson,ZA, Beilke,JN, Korbutt,GS, Rajotte,RV, Gill,RG: The degree of phylogenetic disparity of islet grafts dictates the reliance on indirect CD4 T-cell antigen recognition for rejection. *Diabetes* 52:1433-1440, 2003

16. Nicolls,MR, Coulombe,M, Beilke,J, Gelhaus,HC, Gill,RG: CD4-dependent generation of dominant transplantation tolerance induced by simultaneous perturbation of CD154 and LFA-1 pathways. *J.Immunol.* 169:4831-4839, 2002

17. Rayat,GR, Johnson,ZA, Beilke,JN, Korbutt,GS, Rajotte,RV, Gill,RG: The degree of phylogenetic disparity of islet grafts dictates the reliance on indirect CD4 T-cell antigen recognition for rejection. *Diabetes* 52:1433-1440, 2003

18. Gill,RG, Coulombe,M: Rejection of pancreatic islet xenografts does not require CD8+ T-lymphocytes. *Transplant.Proc.* 24:2877-2878, 1992

19. Gill,RG, Wolf,L, Daniel,D, Coulombe,M: CD4+ T cells are both necessary and sufficient for islet xenograft rejection. *Transplant.Proc.* 26:1203, 1994

20. Cosmi,L, Liotta,F, Lazzeri,E, Francalanci,M, Angeli,R, Mazzinghi,B, Santarlasci,V, Manetti,R, Vanini,V, Romagnani,P, Maggi,E, Romagnani,S, Annunziato,F: Human CD8+CD25+ thymocytes share phenotypic and functional features with CD4+CD25+ regulatory thymocytes. *Blood* 102:4107-4114, 2003

21. Rice,JC, Bucy,RP: Differences in the degree of depletion, rate of recovery, and the preferential elimination of naive CD4+ T cells by anti-CD4

monoclonal antibody (GK1.5) in young and aged mice. *J.Immunol.* 154:6644-6654, 1995

22. Zhao,Y, Sykes,M: Resistance to monoclonal antibody-induced CD8+ T-cell depletion in thymectomized MHC class II-deficient mice. *Transplantation* 64:489-494, 1997

23. Thomson,AW, Robbins,PD: Tolerogenic dendritic cells for autoimmune disease and transplantation. *Ann.Rheum.Dis.* 67 Suppl 3:iii90-iii96, 2008

24. Cobbold,SP: Regulatory T cells and transplantation tolerance. *J.Nephrol.* 21:485-496, 2008

25. Kosiewicz,MM, Alard,P: Tolerogenic antigen-presenting cells: regulation of the immune response by TGF-beta-treated antigen-presenting cells. *Immunol.Res.* 30:155-170, 2004

26. Sato,K, Yamashita,N, Baba,M, Matsuyama,T: Modified myeloid dendritic cells act as regulatory dendritic cells to induce anergic and regulatory T cells. *Blood* 101:3581-3589, 2003

27. Wang,Q, Zhang,M, Ding,G, Liu,Y, Sun,Y, Wang,J, Zhang,W, Fu,Z, Cao,X: Anti-ICAM-1 antibody and CTLA-4Ig synergistically enhance immature dendritic cells to induce donor-specific immune tolerance in vivo. *Immunol.Lett.* 90:33-42, 2003

28. Sun,W, Wang,Q, Zhang,L, Liu,Y, Zhang,M, Wang,C, Wang,J, Cao,X: Blockade of CD40 pathway enhances the induction of immune tolerance by immature dendritic cells genetically modified to express cytotoxic T lymphocyte antigen 4 immunoglobulin. *Transplantation* 76:1351-1359, 2003

29. Li,M, Zhang,X, Zheng,X, Lian,D, Zhang,ZX, Sun,H, Suzuki,M, Vladau,C, Huang,X, Xia,X, Zhong,R, Garcia,B, Min,WP: Tolerogenic dendritic cells transferring hyporesponsiveness and synergizing T regulatory cells in transplant tolerance. *Int.Immunol.* 20:285-293, 2008

30. Markees,TG, Appel,MC, Noelle,RJ, Mordes,JP, Greiner,DL, Rossini,AA: Tolerance to islet xenografts induced by dual manipulation of antigen presentation and co-stimulation. *Transplant.Proc.* 28:814-815, 1996

31. Min,WP, Zhou,D, Ichim,TE, Strejan,GH, Xia,X, Yang,J, Huang,X, Garcia,B, White,D, Dutartre,P, Jevnikar,AM, Zhong,R: Inhibitory feedback loop between tolerogenic dendritic cells and regulatory T cells in transplant tolerance. *J.Immunol.* 170:1304-1312, 2003

32. Kalled,SL, Cutler,AH, Ferrant,JL: Long-term anti-CD154 dosing in nephritic mice is required to maintain survival and inhibit mediators of renal fibrosis. *Lupus* 10:9-22, 2001

33. Pearson,T, Markees,TG, Wicker,LS, Serreze,DV, Peterson,LB, Mordes,JP, Rossini,AA, Greiner,DL: NOD congenic mice genetically protected from autoimmune diabetes remain resistant to transplantation tolerance induction. *Diabetes* 52:321-326, 2003

34. Lunsford,KE, Koester,MA, Eiring,AM, Horne,PH, Gao,D, Bumgardner,GL: Targeting LFA-1 and cd154 suppresses the in vivo activation and development of cytolytic (cd4-Independent) CD8+ T cells. *J.Immunol.* 175:7855-7866, 2005

CHAPTER 5

PD-1/PD-L PATHWAY IS REQUIRED FOR THE INDUCTION AND MAINTENANCE OF TOLERANCE TO NEONATAL PORCINE ISLET XENOGRAFTS BY COMBINED ANTI-LFA-1 AND ANTI-CD154 MONOCLONAL ANTIBODIES

5.1 INTRODUCTION

Transplantation of pancreatic islets into patients with type 1 diabetes results in physiological glycemic control and insulin-independence when sufficient islets are implanted (1;2). However, the widespread clinical application of islet transplantation is limited by *i)* the shortage of human islet donors and *ii)* the long-term use of conventional immunosuppressive drugs that have harmful side effects and potential health risks to the transplanted patients. These factors have excluded the use of islet transplantation in children with type 1 diabetes. Therefore, alternative sources of islets particularly from xenogeneic sources such as pigs and safer anti-rejection therapies are being sought. Recent studies of neonatal porcine islet (NPI) xenotransplantation in small as well as large animal models including non-human primates (3-7) indicate that islet xenotransplantation could be an alternative treatment for human type 1 diabetes. One promising strategy to prevent rejection of islet xenotransplant without using the conventional immune suppressive drugs is the use of biological agents in term of monoclonal antibodies (mAbs) that be developed to target signaling pathways and molecules important for T cell activation. We previously showed that short-term administrations of a combination of mAbs particularly anti-LFA-1 and anti-CD154 mAbs was highly effective in promoting long-term survival of NPI xenografts in B6 mice (7). Our recent data also showed that this combined mAb therapy induced dominant, species- as well as tissue-specific tolerance to NPI xenografts in B6 mice mediated by regulatory T cells (8-10). Characterization of spleen cells from tolerant B6 mouse recipients of NPI that were treated with anti-

LFA-1 and anti-CD154 mAbs showed significantly higher expression of Programmed Death 1 (PD-1) co-inhibitory molecule by spleen cells particularly on $CD4^+$ T cells compared to naïve non-transplanted B6 mice and treated B6 mouse recipients of NPI that eventually rejected their islet grafts (8), suggesting a possible role for PD-1 con-inhibitory pathway in the induction or maintenance of tolerance to NPI xenografts.

PD-1 (CD279) is an inhibitory member of the CD28 co-stimulatory family that was discovered as a membrane bound protein up-regulated in a T cell hybridoma undergoing apoptotic cell death (11). PD-1 is transiently expressed on activated T cell subsets, natural killer cells, B cells and monocytes but constitutively expressed on immature thymocytes, NK cells, and macrophages(12;13). PD-1 binds to two distinct ligands, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) (12;13). While the pattern of expression of PD-L2 is restricted to dendritic cells and macrophages, PD-L1 is constitutively expressed and inducible upon activation on B, T, macrophages and dendritic cells, but it is also expressed on endothelial and epithelial cells (13). PD-1 interaction with its ligands provides a negative signal when transmitted just at the same time with TCR signals but not in the absence of TCR signaling (12;13). It was shown that PD-1 pathway is important in the process of graft rejection or induction of tolerance in different models of transplantation such as murine skin (14), cardiac (15;16) allografts, and islet allo- (17) and xenografts (18) and moreover in a model of graft versus host disease (19). It was shown that using a PD-L1 fusion protein which stimulates activated PD-1 expressing T cells can promote allograft

survival in murine models of cardiac (20), corneal (21), and islet (17) transplantation. With regards to autoimmunity, it was shown that PD-1-deficient mice develop different types of autoimmune disorders indicating that the PD-1/PD-L pathway is implicated in the regulation of peripheral tolerance (22;23). Further, it was shown that blockade of the PD-1/PD-L1 can accelerate the progression and severity of both autoimmune diabetes and experimental autoimmune encephalomyelitis (24-29).

In the current study our aim was to determine the role of PD-1 pathway in the induction and maintenance of tolerance to NPI xenograft induced by a combination of anti-LFA-1 and anti-CD154 mAbs. Our results show that blockade of PD-1/PD-L interaction using long-term administration of anti-PD-1 mAb not only prevent induction of tolerance but also can break tolerance to NPI xenografts induced by a combination of anti-LFA-1 and anti-CD154 mAbs.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Six to 8-week-old male immune-competent B6 (C57BL/6J, H-2^b) and immune-deficient B6 *rag-/-* (B6.129S7-$Rag1^{tm1Mom}$/J, H-2^b) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and were used as recipients. These mice were rendered diabetic by a single intraperitoneal (i.p.) injection of streptozotocin (Sigma, St Louis, MO, USA) at a dose of 180 or 175 mg/kg body weight for B6 or B6 *rag-/-* mice 4-6 days before transplantation, respectively. Blood samples were obtained from the tail vein and glucose levels were monitored using a Precision glucose meter (ONETOUCH, Ultra, Lifescan,

Milpitas, CA, USA). All diabetic mice had two consecutive non-fasting blood glucose levels (BGLs) >20 mmol/l prior to islet transplantation. All mice were fed standard laboratory food and cared for according to the guidelines established by the Canadian Council on Animal Care Committee. Three-day-old Duroc cross neonatal pigs (>1.5 kg body weight) were purchased from the University of Alberta (Edmonton, Alberta, Canada) as islet donors.

5.2.2 Islet isolation and transplantation

Neonatal porcine islets (NPI) were isolated as previously described (6). Neonatal pigs were anesthetized with halothane and subjected to laparotomy and exsanguination. The pancreas was removed, placed in Hanks' balanced salt solution (HBSS, Sigma, St Louis, MO, USA) cut into small pieces, and digested with 2.5 mg/ml collagenase (clostridiopeptidase A, type XI, Sigma-Aldrich, St. Louis, MO, USA). Digested tissue was filtered through a 500 μm nylon screen then cultured for 7 days in HAM's F10 medium (GIBCO Laboratories, Grand Island, NY, USA) containing 10 mmol/l glucose, 50 μmol/l isobutylmethylxanthine (ICN Biomedicals, Montreal, Canada), 0.5% bovine serum albumin (fraction V, radioimmunoassay grade; Sigma), 2 mmol/l L-glutamine, 3 mmol/l $CaCl_2$, 10 mmol/l nicotinamide (BDH Biochemical, Poole, England), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C (5% CO_2, 95% air). A total of 2,000 NPI were transplanted under the kidney capsule of diabetic B6 or B6 *rag-/-* mice as previously described (6;8) . Engraftment was considered successful when blood glucose level reached ≤8.5 mmol/l. Graft rejection was defined as the first of 3 consecutive days of hyperglycemia (>12

mmol/l), and rejection was confirmed by histological analysis of the graft. Nephrectomy of the graft-bearing kidney was performed on recipients with long-term graft function to confirm that normoglycemia was due to the islet xenograft.

5.2.3 Monoclonal antibody therapies

Diabetic B6 mice transplanted with NPI were randomly designated to receive i.p. injection of a combination of anti-LFA-1 mAb (KBA; rat IgG2a; prepared as ascites; 200 μg on days 0, 1, 7, 14 post-transplant) and anti-CD154 mAb (MR-1; hamster IgG1; Bio Express, West Lebanon, NH, USA; 250 μg on days –1, 1 and 2 times a week for an additional 4 weeks post-transplant) alone or plus anti-PD-1 mAb based on one of the following regimens: *i)* short-term administration of anti-PD-1 (J43, hamster IgG2; Bio Express, 500 μg on day 0 and 250 μg on days 2, 4, 6, 8 and 10 post-transplantation)(24), *ii)* long-term administration of anti-PD-1 mAb (J43, hamster IgG2; Bio Express, in a total of 8 injections of 500 μg per injection every other day starting day 0 to 14 post-transplantation), or long-term administration of anti-PD-1 mAb (4F10, rat IgG2a, kappa chain, kindly provided by Dr. Rodriguez-Barbosa; Leon, Spain, in a total 8 injections 500 μg per injection every other day starting on day 0 to 14 post-transplantation)(18).

5.2.4 Immunohistological analysis

The presence of insulin-producing beta cells and immune cell infiltrate in the graft were examined in sections of kidneys bearing the islet grafts. Briefly, graft-bearing kidneys were harvested and fixed in 10% buffered formalin solution and embedded in paraffin, then 5 μm sections were stained with guinea pig anti-

porcine insulin primary antibody (1:1000; DAKO laboratories, Mississauga, Ontario, Canada) for 30 min, followed by the addition of biotinylated goat anti-guinea pig IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). Avidin-biotin complex/horseradish peroxidase (ABC/HP; Vector Laboratories, Burlingame, CA, USA) and 3, 3-diaminobenzidinetetrahydrochloride (DAB; BioGenex, San Ramon, CA, USA) were used to detect positive cells (brown color). All paraffin sections were counter-stained with Harris' hematoxylin and eosin.

5.2.5 Detection of anti-porcine antibodies by flow cytometry

Humoral immune response of B6 mouse recipients of NPI was determined by measuring the levels of mouse anti-porcine IgG antibodies in the serum samples of these mice using flow cytometry. Spleen cells ($1x10^6$) obtained from NPI donors were incubated with 1:128 dilutions of mouse serum for 1 h at 37°C, 5% CO_2, and 95% air. After incubation, spleen cells were then washed with PBS and incubated with 1:200 dilutions of FITC-conjugated rat adsorbed goat anti-mouse IgG antibody (Southern Biotechnology Associates, Inc. Birmingham, Alabama, AL, USA) for 1 h at 4°C. The percentage of cells bound to the antibody was detected from single parameter fluorescence histograms on a BD FACS Calibur flow cytometry machine (BD Biosciences Pharmingen, Mississauga, Ontario, Canada) after gating on viable lymphocytes. Controls for this experiment include porcine spleen cells alone and spleen cells incubated with secondary antibody without mouse serum.

5.2.6 Characterization of lymphocytes by flow cytometry.

Spleen cells from naïve non-transplanted B6 and B6 mouse recipients of NPI were isolated by mechanical disruption of spleen tissue between rough edges of the glass slides. Erythrocytes from spleen cell suspension were depleted using red blood cell lyses buffer. Absolute lymphocyte numbers were determined by staining the cells with Trypan blue exclusion dye, and live cells were counted using a hemocytometer. One million spleen cells were incubated with fluorescent conjugated antibodies (1:100 dilution, eBioscience, San Diego, CA, USA) for 30 min at 4^{o}C. Spleen cells were washed two times with PBS and suspended in 300 µl of FACS buffer (2% FBS in PBS). Fluorescence histograms were created using a BD FACS Calibur flow cytometry machine and were used to determine the percentage of positive cells labeled with the corresponding antibodies. Controls for this experiment include unstained spleen cells from the corresponding mice and spleen cells from non-transplanted naïve B6 mice that were either not incubated or incubated with the antibodies.

5.2.7 Statistical analysis

Statistical differences in expression of cell markers and mouse anti-donor IgG antibody levels between groups were determined using nonparametric Mann-Whitney test. Statistical differences in graft survival among groups were analyzed using the Kaplan-Meier Log rank test. All statistical tests were performed using SPSS statistical software, version 13.0 for Windows (Chicago, IL, USA). A p value of less than 0.05 was considered to be statistically significant.

5.3 RESULTS

5.3.1 Simultaneously short-term administration of a combination of anti-LFA-1 and anti-CD154 plus anti-PD-1 mAbs did not prevent induction of tolerance to NPI xenografts.

Since we previously showed that tolerant B6 mice to NPI xenografts have higher expression of PD-1 compared to the non-tolerant B6 mice that eventually rejected their NPI xcenografts and naïve non-transplanted B6 mice (8), we examined whether blockade of PD-1/PD-L interaction using short-term administrations of anti-PD-1 mAb (J43) could prevent the induction of tolerance to NPI xenografts induced by a combination of anti-LFA-1 and anti-CD154 mAbs. It was shown that blockade of the PD-1/PD-L1 using short-term administration of anti-PD-1 mAb (J43) can accelerate the progression and severity of autoimmune diabetes in NOD mice (24). All of the B6 mouse recipients of NPI simultaneously treated with short-term administrations of anti-PD-1 mAb plus a combination of LFA-1 and anti-CD154 mAbs achieved normal blood glucose levels and had prolonged NPI xenograft survival for over 100 days post-transplantation (Table 5.1). Histological analysis of the NPI xenografts from these mice and B6 mice treated with anti-LFA-1 and anti-CD154 mAbs alone showed intact islets of insulin producing cells and no immune cell infiltration (Figure 5.1A and B, respectively). These observations were comparable to what was seen in the NPI xenograft harvested from immune-deficient B6 *rag-/-* mice transplanted with same islets (Figure 5.1C). In addition, the amount of anti-porcine antibodies production in B6 mouse recipients of NPI that were treated with anti-LFA-1 and

anti-CD154 mAbs alone or plus short-term administrations of anti-PD-1 mAb (Figure 5.2A and B, respectively) were comparable to the amount that was detected in naive non-transplanted B6 mice (Figure 5.2C).

Table 5.1: Survival of NPI xenografts in B6 mice treated with a combination of anti-LFA-1, anti-CD154 and anti-PD-1 mAbs.

Group	n	Graft survival (days post-transplantation)*	Number of mice that achieved normoglycemia
anti-LFA-1+anti-CD154 mAbs	8	>100 (x8)	8
anti-LFA-1+anti-CD154 mAbs plus anti-PD-1 mAb (J43, short-term)	8	>100 (x8)	8
anti-LFA-1+anti-CD154 mAbs plus anti-PD-1 mAb (4F10, long-term) *, †	8	0(x8)	0
anti-LFA-1+anti-CD154 mAbs plus anti-PD-1 mAb (J43, long-term) *	8	0 (x4), >100 (x4)	4

*$p<0.05$ vs. the group of mice treated with short-term administration of anti-PD-1 (J43)
†$p<0.05$ vs. the group of mice treated with long-term administration of anti-PD-1 (J43)

Based on these results targeting PD-1/PD-L interaction using short-term administration of anti-PD-1 mAbs can not prevent induction of tolerance to NPI xenografts by a combination of anti-LFA-1 and anti-CD154 mAbs.

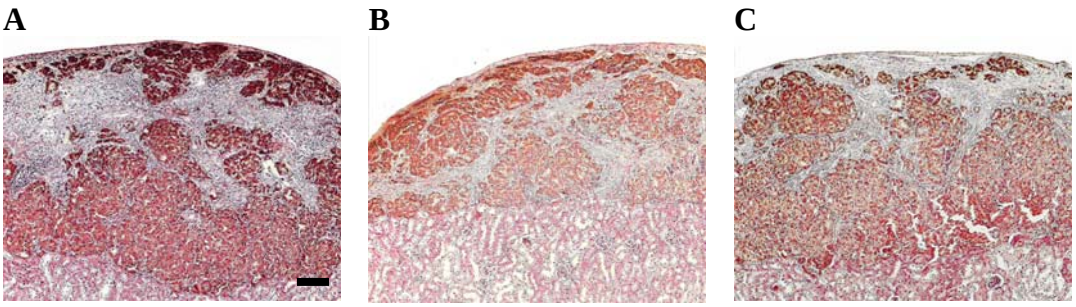

Figure 5.1: Representative NPI xenografts in B6 mice treated with a combination of anti-LFA-1 and anti-CD154 mAbs alone or plus short-term administrations of anti-PD-1 mAb. Islet grafts from B6 mouse recipients treated with tolerogenic regiman alone (A) or plus short-term administration of anti-PD-1 (clone J43) mAb (B) contained intact islets with abundant insulin-positive cells (brown stain). NPI xenografts from B6 *rag-/-* mice transplanted with the same NPI xenograft (C). Scale bar represents 100 μm.

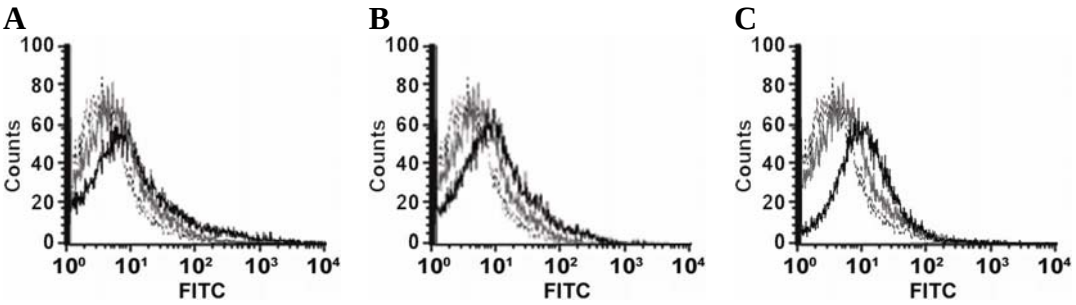

Figure 5.2: Humoral responses of B6 mouse recipients of NPI treated with a combination of LFA-1 and anti-CD154 mAbs alone or plus short-term administrations of anti-PD-1 mAb. The levels of IgG antibodies from tolerant B6 mouse recipients of NPI treated with a combination of anti-LFA-1 and anti-CD154 mAbs alone (5.1%-8.3%, n=8, A) or plus short-term administration of anti-PD-1 (J43) mAb (5.6 % - 8.5%, n=8, B) were comparable to the amount of anti-porcine IgG antibodies detected in the serum of naive non-transplanted B6 mice (4.9%-7.3%, n=5, C). Representative histograms are shown and controls for this experiment consisted of unstained (dashed black line) and secondary antibody without serum (solid gray line).

5.3.2 Long-term administration of anti-PD-1 mAb in combination with anti-LFA-1 and anti-CD154 mAbs can prevent the induction of tolerance to NPI xenografts.

We next assessed whether long-term administrations of a combination of anti-PD-1 mAb can prevent induction of tolerance to NPI xenografts by a combination of anti-LFA-1 and anti-CD154 mAbs. We used the regimen that was found effective in reversing the protective effect of anti-CD154 mAb administration in a model of concordant rat to mouse xenogeneic islets (18). None of the mice simultaneously treated with long-term administration of anti-PD-1 mAb (4F10) plus a combination of anti-LFA-1 and anti-CD154 mAbs achieved normoglycemia, indicating of NPI xenograft rejection (Table 5.1). Histological analysis of the NPI xenografts harvested from the B6 mice treated with a combined anti-LFA-1 and anti-CD154 mAbs plus long-term administration of anti-PD-1 (4F10) revealed absence of insulin positive cells in the graft site and presence of abundant immune cells infiltrates (Figure 5.3A). In another set of experiment we also tried long-term administrations of anti-PD-1 (J43) which is commercially available, plus a combination of anti-LFA-1 and anti-CD154 mAbs to compare the effect of this anti-PD-1 mAb (J43) with previously used anti-PD-1 mAb (4F10) in preventing induction of tolerance to NPI xenografts by a combination of anti-LFA-1 and anti-CD154 mAbs. Our data showed that 4 out of 8 B6 mice treated with long-term administration of anti-PD-1 (J43) plus a combination of anti-LFA-1 and anti-CD154 mAbs achieved normoglycemia within 5-8 weeks post-transplantation and maintained normoglycemia for over

100 days post-transplantation (Table 5.1). NPI xenograft function from these mice was confirmed by removing the graft-bearing kidney, which was followed by a rapid return to hyperglycemia. Immunohistochemical staining of NPI xenografts from the mice with long-term surviving graft revealed some insulin-producing β cells and mononuclear cell infiltration (Figure 5.3B). These results demonstrate that PD-1/PD-L pathway play an important role in the induction of tolerance to NPI xenografts (Table 5.1). The levels of anti-porcine IgG antibodies in all of the mice in these groups (Figure 5.4 C and D, respectively) were significantly higher than the levels of anti-porcine IgG antibodies detected in B6 mouse recipients of NPI that received a combination of ant-LFA-1 and anti-CD154 mAbs and naïve non-transplanted B6 mice (Figure 5.3B and C, respectively).

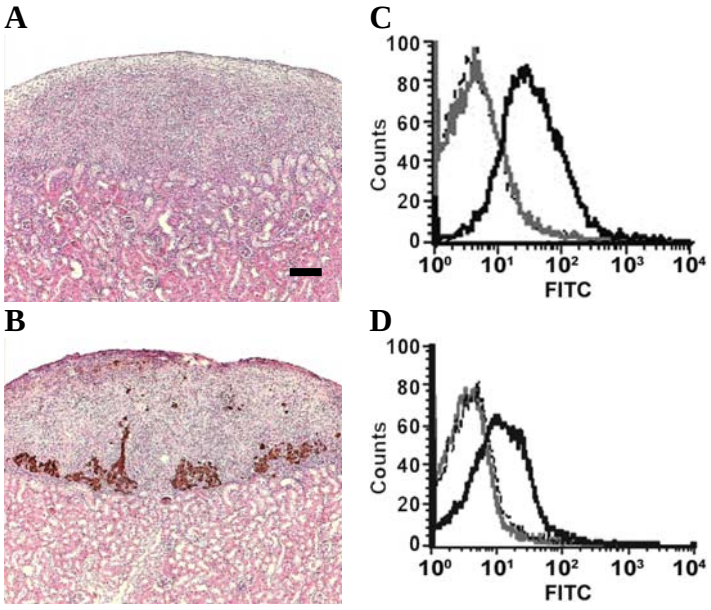

Figure 5.3: Representative of NPI xenografts and humoral responses of mice treated with a combination of anti-LFA-1 and anti-CD154 mAbs plus long-term administration of anti-PD-1 (4F10 or J43) mAbs. NPI xenografts in B6 mouse recipients treated with a combination of anti-LFA-1 nad anti-CD154 mAbs plus long-term administration of anti-PD-1 mAb (either 4F10 or J43) revealed no

or few insulin positive cells in the graft site, respectively and abundant of immune cell infiltrates (A and B, respectively). Scale bar represents 100 μm. The levels of anti-porcine IgG antibodies from B6 mice treated with a combination of anti-LFA-1 and anti-CD154 mAbs plus long-term administration of anti-PD-1 mAbs (33.65-46.56% for 4F10 treated group, and 16.58-23.53% for J43 treated group, n=8 in each group) were significantly higher than the levels of anti-porcine IgG antibody detected in the serum of B6 mouse recipients of NPI treated with a combination of anti-LFA-1 and anti-CD154 mAbs alone (5.1%-8.3%, n=8, Figure 5.2A). Controls for this experiment consisted of unstained (dashed black line) and secondary antibody without serum (solid gray line).

5.3.3 Combined anti-LFA-1 and anti-CD154 mAb therapy result in PD-1$^+$ cell-mediated tolerance to NPI xenografts.

To better define the role of PD-1 pathway in the maintenance of tolerance induced by combined anti-LFA-1 and anti-CD154 mAbs, a group of tolerant B6 mice was treated with long-term administration of anti-PD-1 mAb (J43) beginning at 150 days post-transplantation. All normoglycemic recipient mice became diabetic by 27 days post-injection of anti-PD-1 mAb (n=8, Table 5.2). NPI xenografts from these mice had infiltrating immune cells and no insulin-positive cells remaining in the grafts (Figure 5.4A). The levels of mouse anti-porcine IgG antibodies in these mice were significantly higher (Figure 5.4B) than those detected in tolerant B6 mice that did not received anti-PD-1 mAb treatment and naïve non-transplanted B6 mice (Figure 5.2B and C, respectively). These results indicate that PD-1/PD-L interaction is required for maintanence of tolerance to NPI xenografts induced by acombination of anti-LFA-1 and anti-CD154 mAbs.

Table 5.2: NPI xenograft survival in tolerant B6 mice treated with long-term administrations of anti-PD-1 mAb.

Group	n	Graft Survival (Days post-transplantation)
No treatment	5	>250 (x5)
anti-PD-1 mAb	8	166, 168, 171(x2), 173(x3),177*

* $p<0.01$ vs. untreated mice

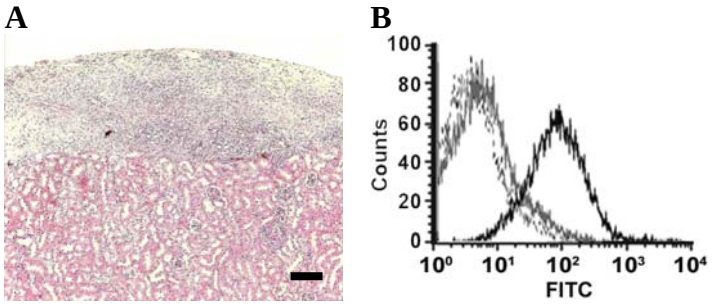

Figure 5.4: Representative NPI xengrafts and humoral immune responses of tolerant B6 mice treated with anti-PD-1 mAb on day 150 post-transplnatation. All tolerant B6 mice (n=8) that received long-term administrations of anti-PD-1 mAb beginning at 150 days post-transplantation became diabetic by day 27 post-injection. NPI xenografts had infiltrating immune cells and absence of insulin-positive cells (A). Scale bar represents 100 μm. The levels of mouse anti-porcine IgG antibodies in these mice were significantly ($p<0.001$) higher (46.36±3.61%, n=8, B) compared to the levels detected in tolerant B6 mice that were not treated with anti-PD-1 mAb and naïve non-transplanted B6 mice (Figure 5.3A and C, respectively). Controls for this experiment consisted of unstained spleen cells (dashed black line) and spleen cells incubated with secondary antibody without serum (solid gray line).

Further characterization of spleen cells from tolerant B6 mice using flow cytometery indicated that majority (90.39±0.88%) of $CD4^+CD25^+$ co-expressed PD-1foxp3 markers (Figure 5.5A). On the other hand, majority of $CD4^+PD\text{-}1^+$ cells (87.80±1.29%) from tolerant B6 mice co-express CD25foxp3 markers

(Figure 5.5B). Significantly higher frequencies of $CD4^+CD25^+foxp3^+PD\text{-}1^+$ T cells were detected tolerant B6 mice compared to those cells detected in naïve non-transplanted B6 mice (Figure 5.5 A and B).

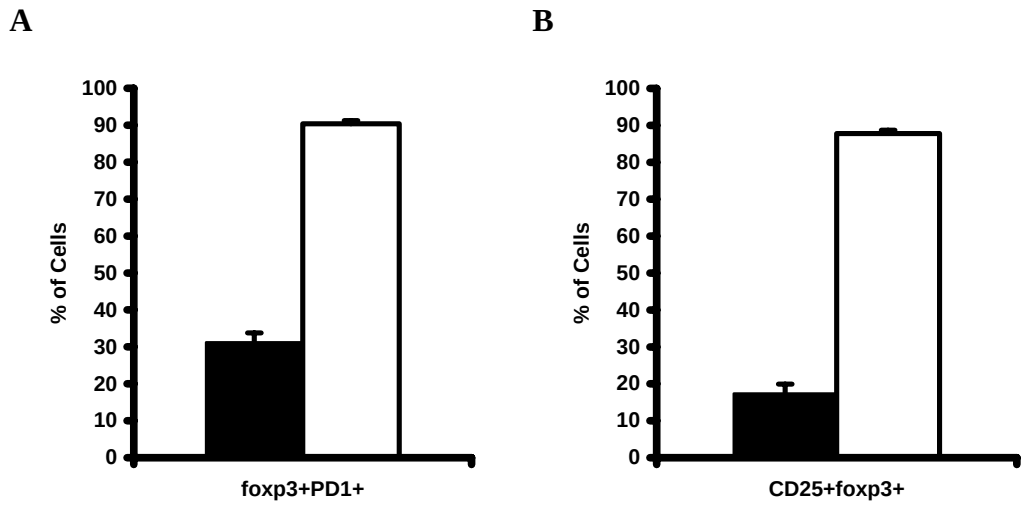

Figure 5.5: High frequency of $CD4^+CD25^+PD\text{-}1^+foxp3^+$ cells were detected in tolerant B6 mice. Tolerant spleen cells co-expressed significantly higher percentage of PD-1foxp3 markers (white bars, 90.39±0.88%, $p<0.001$, n=5) on $CD4^+CD25^+$ gated cells compared to same cell population from naïve non-transplanted B6 mice (black bars, 31.20±2.54%, n=5, A). Higher percentage of CD25foxp3 markers (87.80±1.29%, n=5, $p<0.001$) were detected on $CD4^+PD\text{-}1^+$ gated cells from tolerant B6 mice than the same cell populations from naïve non-transplanted B6 mice (17.38±1.41%, n=5, B).

Collectively, these results suggest that $CD4^+CD25^+foxp3^+PD1^+$ regulatory T cells play an important role in the maintenance of tolerance to NPI xenografts induced by a combination of anti-LFA-1 and anti-CD154 mAbs.

5.4 DISCUSSION

We showed previously that a combination of anti-LFA-1 and anti-Cd154 mAbs is highly effective in induction of tolerance to NPI xenografts mediated by

regulatory T cells (8-10). Significantly lower expression of coinhibtiory molecules particularly PD-1 expression by spleen cells isolated from the B6 mouse recipients of NPI treated with a combination of anti-LFA-1 and anti-CD154 mAbs which eventually rejected their NPI xenografts as well as significantly higher expression of PD-1 particularly on $CD4^+$ T cells of tolerant mice suggesting a possible role of PD-1 in the induction and maintenance of tolerance to NPI xenografts (8). Therefore the purpose of this study was to investigate the role of PD-1/PD-L interaction on induction and maintenance of tolerance to NPI xenografts induced by combined anti-LFA-1 and anti-CD154 mAbs. Our data show that PD-1/PD-L pathway is important in induction of tolerance to NPI xenografts as 100% and 50% of mice treated with long-term administration of anti-PD-1, clone 4F10 and J43 respectively did not achieve normoglycemia and rejected their islet xenografts. Abundant immune cells infiltrate in the NPI xenografts harvested from the normoglycemic mice treated with long-term administration of anti-PD-1 mAb (J43) provided evidence of NPI xenograft rejection. The difference seen between these two groups of mice treated with long-term administrations of anti-PD-1 (clone 4F10 and J43) could be due to the difference in the strength of negative signals considering the point that these two mAbs target the same epitope (18). We also showed that a short-term administration of anti-PD-1 mAb (clone J43) is not enough to prevent induction of tolerance to NPI xenografts induced by a combination of anti-LFA-1 and anti-CD154 mAbs. The importance of PD-1/PD-L pathway have previously been reported in the induction and maintenance of peripheral tolerance(10;14;15;17;30-

34). We showed previously the role of $CD4^+CD25^+foxp3^+$ cells in maintaining tolerance to NPI xenografts by depleting of $CD25^+$ cells from the periphery (8) and by adoptively transferring a combination of spleen cells from tolerant and naïve B6 mice into immune-deficient B6 *rag-/-* mouse recipients of NPI to test for the presence of function of regulatory T cells (8;9). We addressed here the importance of PD-1-dependent mechanism in the maintenance of tolerance to NPI xenografts by targeting the PD-1 pathway using long-term administration of anti-PD-1 mAb. Similar time of graft survival between the tolerant mice treated with anti-PD-1 mAb (21.5 ± 1.2 days post-injection of anti-PD-1 mAb) and anti-CD25 mAb (25.3 ± 2.5 days post-injection of anti-CD25 mAb (8))could be a possible reason of co-expression of these molecules on a same cell population. Further characterization of the spleen cells from tolerant mice indicated that >90% either $CD4^+CD25^+$ or $CD4^+PD\text{-}1^+$ cells also co-expressing $PD\text{-}1^+foxp3^+$ or $CD25^+foxp3^+$ cell markers, respectively. Taken together our data showed the critical role of PD-1/PD-L pathway in the induction and maintenance of tolerance to NPI xenografts induced by a combination of anti-LFA-1 and anti-CD14 mAbs.

5.4 REFERENCES

1. Ryan,EA, Lakey,JR, Rajotte,RV, Korbutt,GS, Kin,T, Imes,S, Rabinovitch,A, Elliott,JF, Bigam,D, Kneteman,NM, Warnock,GL, Larsen,I, Shapiro,AM: Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes* 50:710-719, 2001

2. Shapiro,AM, Lakey,JR, Ryan,EA, Korbutt,GS, Toth,E, Warnock,GL, Kneteman,NM, Rajotte,RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N.Engl.J.Med.* 343:230-238, 2000

3. Cardona,K, Korbutt,GS, Milas,Z, Lyon,J, Cano,J, Jiang,W, Bello-Laborn,H, Hacquoil,B, Strobert,E, Gangappa,S, Weber,CJ, Pearson,TC, Rajotte,RV, Larsen,CP: Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat.Med.* 12:304-306, 2006

4. Kin,T, Korbutt,GS, Kobayashi,T, Dufour,JM, Rajotte,RV: Reversal of diabetes in pancreatectomized pigs after transplantation of neonatal porcine islets. *Diabetes* 54:1032-1039, 2005

5. Kobayashi,T, Harb,G, Rayat,GR: 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, 2005

6. Korbutt,GS, Elliott,JF, Ao,Z, Smith,DK, Warnock,GL, Rajotte,RV: Large scale isolation, growth, and function of porcine neonatal islet cells. *J.Clin.Invest* 97:2119-2129, 1996

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

8. Arefanian,H, Tredget,EB, Rajotte,RV, Gill,RG, Korbutt,GS, Rayat,GR: Short-Term Administrations of a Combination of Anti-LFA-1 and Anti-CD154 Monoclonal Antibodies Induces Tolerance to Neonatal Porcine Islet Xenografts. *Diabetes* 10.2337/DB09-0413: 2009

9. Arefanian,H, Mok,CM, Ramji,Q, Rajotte,RV, Gill,RG, Korbutt,GS, Rayat,GR: Cellular Mechanism of Dominant Tolerance to Neonatal Porcine Islet Xenografts Induced by a Combination of Anti-LFA-1 and Anti-CD154 Monoclonal Antibodies In Mice. *Diabetes* 2009

10. Arefanian,H, Tredget,EB, Mok,M, Rajotte,RV, Gill,RG, Korbutt,GS, Rayat,GR: Species and Tissue-Specific Tolerance to Neonatal Porcine Islet Xenografts Induced by A Combination of Anti-LFA-1 and Anti-CD154 Monoclonal Antibodies in C57BL/6J Mice. *Diabetes* 10.2337/DB09-0898: 2009

11. Ishida,Y, Agata,Y, Shibahara,K, Honjo,T: Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J.* 11:3887-3895, 1992

12. Sharpe,AH, Wherry,EJ, Ahmed,R, Freeman,GJ: The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat.Immunol.* 8:239-245, 2007

13. Agata,Y, Kawasaki,A, Nishimura,H, Ishida,Y, Tsubata,T, Yagita,H, Honjo,T: Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int.Immunol.* 8:765-772, 1996

14. Sandner,SE, Clarkson,MR, Salama,AD, Sanchez-Fueyo,A, Domenig,C, Habicht,A, Najafian,N, Yagita,H, Azuma,M, Turka,LA, Sayegh,MH: Role of the programmed death-1 pathway in regulation of alloimmune responses in vivo. *J.Immunol.* 174:3408-3415, 2005

15. Ito,T, Ueno,T, Clarkson,MR, Yuan,X, Jurewicz,MM, Yagita,H, Azuma,M, Sharpe,AH, Auchincloss,H, Jr., Sayegh,MH, Najafian,N: Analysis of the role of negative T cell costimulatory pathways in CD4 and CD8 T cell-mediated alloimmune responses in vivo. *J.Immunol.* 174:6648-6656, 2005

16. Tao,R, Wang,L, Han,R, Wang,T, Ye,Q, Honjo,T, Murphy,TL, Murphy,KM, Hancock,WW: Differential effects of B and T lymphocyte attenuator and programmed death-1 on acceptance of partially versus fully MHC-mismatched cardiac allografts. *J.Immunol.* 175:5774-5782, 2005

17. Gao,W, Demirci,G, Strom,TB, Li,XC: Stimulating PD-1-negative signals concurrent with blocking CD154 co-stimulation induces long-term islet allograft survival. *Transplantation* 76:994-999, 2003

18. Mai,G, del Rio,ML, Tian,J, Ramirez,P, Buhler,L, Rodriguez-Barbosa,JI: Blockade of the PD-1/PD-1L pathway reverses the protective effect of anti-CD40L therapy in a rat to mouse concordant islet xenotransplantation model. *Xenotransplantation* 14:243-248, 2007

19. Blazar,BR, Carreno,BM, Panoskaltsis-Mortari,A, Carter,L, Iwai,Y, Yagita,H, Nishimura,H, Taylor,PA: Blockade of programmed death-1 engagement accelerates graft-versus-host disease lethality by an IFN-gamma-dependent mechanism. *J.Immunol.* 171:1272-1277, 2003

20. Ozkaynak,E, Wang,L, Goodearl,A, McDonald,K, Qin,S, O'Keefe,T, Duong,T, Smith,T, Gutierrez-Ramos,JC, Rottman,JB, Coyle,AJ, Hancock,WW: Programmed death-1 targeting can promote allograft survival. *J.Immunol.* 169:6546-6553, 2002

21. Watson,MP, George,AJ, Larkin,DF: Differential effects of costimulatory pathway modulation on corneal allograft survival. *Invest Ophthalmol.Vis.Sci.* 47:3417-3422, 2006

22. Nishimura,H, Okazaki,T, Tanaka,Y, Nakatani,K, Hara,M, Matsumori,A, Sasayama,S, Mizoguchi,A, Hiai,H, Minato,N, Honjo,T: Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* 291:319-322, 2001

23. Nishimura,H, Nose,M, Hiai,H, Minato,N, Honjo,T: Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity.* 11:141-151, 1999

24. Ansari,MJ, Salama,AD, Chitnis,T, Smith,RN, Yagita,H, Akiba,H, Yamazaki,T, Azuma,M, Iwai,H, Khoury,SJ, Auchincloss,H, Jr., Sayegh,MH: The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J.Exp.Med.* 198:63-69, 2003

25. Khoury,SJ, Sayegh,MH: The roles of the new negative T cell costimulatory pathways in regulating autoimmunity. *Immunity.* 20:529-538, 2004

26. Salama,AD, Chitnis,T, Imitola,J, Ansari,MJ, Akiba,H, Tushima,F, Azuma,M, Yagita,H, Sayegh,MH, Khoury,SJ: Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *J.Exp.Med.* 198:71-78, 2003

27. Martin-Orozco,N, Wang,YH, Yagita,H, Dong,C: Cutting Edge: Programmed death (PD) ligand-1/PD-1 interaction is required for CD8+ T cell tolerance to tissue antigens. *J.Immunol.* 177:8291-8295, 2006

28. Keir,ME, Liang,SC, Guleria,I, Latchman,YE, Qipo,A, Albacker,LA, Koulmanda,M, Freeman,GJ, Sayegh,MH, Sharpe,AH: Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J.Exp.Med.* 203:883-895, 2006

29. Fife,BT, Guleria,I, Gubbels,BM, Eagar,TN, Tang,Q, Bour-Jordan,H, Yagita,H, Azuma,M, Sayegh,MH, Bluestone,JA: Insulin-induced remission in new-onset NOD mice is maintained by the PD-1-PD-L1 pathway. *J.Exp.Med.* 203:2737-2747, 2006

30. Izawa,A, Yamaura,K, Albin,MJ, Jurewicz,M, Tanaka,K, Clarkson,MR, Ueno,T, Habicht,A, Freeman,GJ, Yagita,H, Abdi,R, Pearson,T, Greiner,DL, Sayegh,MH, Najafian,N: A novel alloantigen-specific CD8+PD1+ regulatory T cell induced by ICOS-B7h blockade in vivo. *J.Immunol.* 179:786-796, 2007

31. Zha,Y, Blank,C, Gajewski,TF: Negative regulation of T-cell function by PD-1. *Crit Rev.Immunol.* 24:229-237, 2004

32. Guleria,I, Khosroshahi,A, Ansari,MJ, Habicht,A, Azuma,M, Yagita,H, Noelle,RJ, Coyle,A, Mellor,AL, Khoury,SJ, Sayegh,MH: A critical role for the programmed death ligand 1 in fetomaternal tolerance. *J.Exp.Med.* 202:231-237, 2005

33. Aramaki,O, Inoue,F, Takayama,T, Shimazu,M, Kitajima,M, Ikeda,Y, Okumura,K, Yagita,H, Shirasugi,N, Niimi,M: Various costimulatory pathways are essential for induction of regulatory cells by intratracheal delivery of alloantigen. *Transplant.Proc.* 37:1934-1936, 2005

34. Totsuka,T, Kanai,T, Makita,S, Fujii,R, Nemoto,Y, Oshima,S, Okamoto,R, Koyanagi,A, Akiba,H, Okumura,K, Yagita,H, Watanabe,M: Regulation of murine chronic colitis by CD4+. *Eur.J.Immunol.* 35:1773-1785, 2005

CHAPTER 6

COMBINATION OF ANTI-CD4 WITH ANTI- LFA-1 AND ANTI- CD154 MONOCLONAL ANTIBODIES PROMOTES LONG-TERM SURVIVAL AND FUNCTION OF NEONATAL PORCINE ISLET XENOGRAFTS IN SPONTANEOUSLY DIABETIC NOD MICE

A version of this chapter has been accepted for publication. Arefanian,H, et al. Cell Transplant. 16:787-798, 2007

6.1 INTRODUCTION

Type 1 diabetes mellitus (T1DM) is caused by the autoimmune destruction of pancreatic islet β cells, which are required for the production of insulin (1). It is an autoimmune disease that typically occurs in childhood and adolescence and has been estimated to account for 5% to 10% of all diagnosed cases of diabetes (2). The non-obese diabetic (NOD) mouse has been used extensively as an animal model of human T1DM. These mice spontaneously begin to develop diabetes after 12 weeks of age and by 30 weeks, approximately 90% of female mice have diabetes (3). Similar in humans, the development of diabetes in NOD mice has been attributed to the autoreactive T cells that infiltrate the pancreatic islets and specifically destroy the insulin-producing β cells. The development of insulitis and diabetes in NOD mice has been shown to be dependent on both $CD4^+$ and $CD8^+$ T cells and facilitated by B cells (3,4). In addition to T1DM, NOD mice develop spontaneous autoimmunity directed at other targets namely the thymus, adrenal gland, salivary glands, thyroid, testis, nuclear components, and red blood cells (3). They also exhibit multiple immune system abnormalities including defect in antigen presenting cells, regulation of T cell repertoires, and natural killer cell function. Pancreatic islet transplantation is an attractive alternative treatment option for patients with T1DM as it has the potential to safely restore euglycemia (5). Islet transplantation also allows stable glucose homeostasis in type 1 diabetic patients without exogenous insulin administration, thus avoiding the risk of developing life-threatening complications associated with diabetes (2). However, the shortage of human cadaveric organs available for

transplantation and chronic administration of harmful immunosuppressive drugs (2,5) to prevent rejection are among the major barriers that currently limit the widespread clinical application of islet transplantation. For islet transplantation to be widely applicable to individuals with diabetes, alternative sources of islets and safer anti-rejection strategies that will induce transplantation tolerance are needed. One potential solution to the shortage of human islets is the utilization of islets from animals, particularly the pig. Animal size, availability, ease of husbandry, suitability for genetic manipulation, and the structural homology of porcine and human insulin all make the pig a strong candidate source for islets (6). Adult porcine islets have been extensively studied (7-10), but in our experience, adult porcine islets are difficult to isolate and maintain in culture. This constraint has led us to evaluate the potential of neonatal porcine islets (NPI) as an alternative islet source for clinical transplantation. NPI are an attractive alternative source of insulin-producing cells, because they are easy to isolate and maintain in culture, have considerable capacity for growth and differentiation, and can correct hyperglycemia in immune-deficient animals (11-14) and in large animals (15,16) including non-human primates (4). Our group and others have shown that NPI xenografts are rapidly rejected primarily by $CD4^+$ T cells via the host MHC class II - restricted indirect pathway of antigen recognition (12,17,18). One strategy that was effective in preventing rejection of NPI xenografts is the strategic combination of monoclonal antibodies (mAbs) for blocking a variety of cell surface molecules involved in T-cell activation. We showed that short-term administration of a combination of anti-LFA-1 and anti-CD154 mAbs promotes

long-term survival of NPI xenografts in immune-competent B6 mice (19). Others have also demonstrated that this combined mAb therapy was effective in preventing the rejection of islet allografts in either chemically induced (20) or spontaneously diabetic NOD (21) mice and can induce a robust form of dominant allograft tolerance (20). Therefore, the aim of this study was to determine the efficacy of combined anti-LFA-1 and anti-CD154 mAb therapy in preventing rejection of NPI xenografts in autoimmune prone NOD mice. In addition, we determined the efficacy of anti-CD4 mAb alone or in combination with anti-LFA-1 and anti-CD154 mAbs in promoting the survival of NPI xenografts in NOD mice since $CD4^+$ T cells play a pivotal role in the onset of diabetes and generation of anti-graft responses, particularly against xenografts (22-29).

6.2 MATERIALS AND METHODS

6.2.1 Animals

Seventeen to 35 weeks-old spontaneously diabetic female NOD mice (NOD/MrkTacfBR, H-2^{g7}) from Taconic farms (Germantown, NY, USA) and NOD.129S7 (B6)-*Rag1*^{tm1Mom}/J (NOD *Rag-/-*), from the Jackson Laboratory (BarHarbor, MD, USA) were used as recipients. NOD *rag-/-* mice were rendered diabetic by a single intraperitoneal injection of 185 mg/kg streptozotocin (Sigma, St Louis, MO, USA). NOD mice were screened weekly for diabetes and mice with three consecutive non-fasting blood glucose levels ≥25.0 mmol/l within 7 days prior to islet transplantation were subsequently used as islet transplant recipients. Glucose levels were measured from blood samples obtained from the tail vein using a Precision glucose meter (ONETOUCH, Ultra, Lifescan, Milpitas,

CA, USA). All mice were housed under specific pathogen-free conditions and were fed standard laboratory food and cared for according to the guidelines established by the Health Sciences Animal Policy and Welfare Committee at the University of Alberta.

6.2.2 Islet isolation and transplantation

One to 3-day-old Landrace-Yorkshire (1.5 to 2.0 kg body weight) neonatal pigs were purchased from the University of Alberta farm and were used as islet donors. NPI were isolated as previously described (11). Briefly, neonatal pigs were anesthetized with halothane and subjected to laparotomy and exsanguination. The pancreas was removed, placed in Hanks' balanced salt solution (HBSS, Sigma, St Louis, MO, USA) cut into small pieces, and digested with 2.5 mg/ml collagenase (clostridiopeptidase A, type XI, Sigma-Aldrich, St. Louis, MO, USA). Digested tissue was filtered through a 500 μm nylon screen then cultured for 7 days in HAM's F10 medium (GIBCO Laboratories, Grand Island, NY, USA) containing 10 mmol/l glucose, 50 μmol/l isobutylmethylxanthine (ICN Biomedicals, Montreal, Canada), 0.5% bovine serum albumin (fraction V, radioimmunoassay grade; Sigma), 2 mmol/l L-glutamine, 3 mmol/l $CaCl_2$, 10 mmol/l nicotinamide (BDH Biochemical, Poole, England), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C (5% CO_2, 95% air). After 7 days of culture, NPI were counted for transplantation and a total of 2,000 NPI were transplanted under the left kidney capsule of spontaneously diabetic NOD mice as described previously (11). In brief, spontaneously diabetic NOD mice were anesthetized by inhalational isoflurane and the left flank was shaved and sterilized with 100%

ethanol. A flank incision was made lateral to the left paraspinal muscles and the peritoneum was sharply divided to expose the left kidney. A sterile cotton swab was utilized to expose and produce the left kidney from the incision. A small incision was made in the kidney capsule with a 27-gauge needle and a subcapsular pocket was expanded with the use of a sterile Pasteur pipette. Aliquots of 2,000 NPI were aspirated into polyethylene (PE-50) tubing, pelleted by centrifugation and placed within the subcapsular pocket with the aid of a micromanipulator syringe. Once the tubing was removed, the kidney capsule was cauterized with a disposable high-temperature cautery pen (Aaron Medical Industries, St. Petersburg, FL, USA). Euglycemia was defined as blood glucose values <10 mmol/l and graft rejection was defined as the first of three consecutive days of hyperglycemia >10.0 mmol/l which was confirmed by histological analysis of the graft.

6.2.3 Monoclonal antibody therapies

Spontaneously diabetic NOD mice transplanted with NPI were randomly designated to receive intraperitoneal injection of the following mAb treatments: 1) Short-term administration of anti-LFA-1 mAb (KBA; rat IgG2a; 200 μg on days 0, 1, 7, 14 post-transplant) plus anti-CD154 mAb (MR-1; hamster IgG1; Bio Express, West Lebanon, NH, USA; 250 μg on days –1, 1 and 2 times a week for an additional 4 weeks post-transplant); 2) Double dose administration of anti-LFA-1 mAb (400 μg on days 0, 1 and 200 μg on days 7, 14 post-transplant) plus anti-CD154 mAb; 3) Long-term administration of anti-LFA-1 mAb (200 μg on days 0, 1, 7, 14 and once a week for an additional 8 weeks post-transplant) and

anti-CD154 mAb (250 μg on days –1, 1 and 2 times a week for an additional 10 weeks post-transplant), 4) anti-CD4 mAb alone (GK1.5, rat IgG2b; Bio Express, 20 mg/kg on days -4, -2, 0, 2 and 7 post-transplant), and finally 5) Triple therapy consisting of a combination of short-term administration of anti-CD4, anti-LFA-1, and anti-CD154 mAbs.

6.2.4 Immunohistological analysis

To examine the presence of insulin-producing β cells and immune cell infiltrate in the graft, kidneys bearing NPI xenograft were harvested and divided in two sections on the day of rejection or at the end of the study (>100 days post-transplantation). One half of the kidney was fixed in 10% buffered formalin solution and embedded in paraffin, then 5 μm sections were stained with guinea pig anti-insulin primary antibody (1:1000; DAKO laboratories, Mississauga, Ontario, Canada) for 30 min, followed by the addition of biotinylated goat anti-guinea pig IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). Avidin-biotin complex/horseradish peroxidase (ABC/HP; Vector Laboratories) and 3, 3-diaminobenzidinetetrahydrochloride (DAB; BioGenex, San Ramon, CA, USA) were used to detect positive cells (brown color). All paraffin-embedded tissue sections were counter-stained with Harris' hematoxylin and eosin.

The other half of the kidney was embedded in OCT compound (Miles Scientific, Naperville, IL, USA) and kept frozen at –80°C to determine the presence of immune cells. Five-micron frozen sections were air dried for 10 min, fixed in acetone for 3 min at 4°C, and then washed in PBS. Non-specific binding

was eliminated by incubating the tissue sections in 2% fetal bovine serum (FBS) in PBS for 20 min. In addition, endogenous avidin and biotin or biotin-binding proteins present in the sections were also eliminated using the avidin/biotin blocking kit (Vector Laboratories). Rat anti-mouse CD4 antibody (1:100; BD Biosciences Pharmingen, Ontario, Canada), rat anti-mouse CD8a antibody (1:100; BD Biosciences Pharmingen), rat anti-mouse CD19 antibody (1:500; BD Biosciences Pharmingen) or rat anti-mouse CD11b antibody (1:500; BD Biosciences Pharmingen) was applied to designated tissue sections for 30 min at room temperature. Biotinylated goat anti-rat IgG secondary antibody (1:200; Southern Biotechnology Associates, Inc., Birmingham, AL, USA) was added and incubated for 30 min. ABC/HP reagent and DAB were applied to produce a brown color. Sections were then counter-stained with Harris' hematoxylin.

6.2.5 Detection of anti-porcine and autoreactive antibodies

To determine the effect of mAb therapies on the humoral immune response of NOD mice to NPI xenografts, the levels of mouse anti-porcine IgG antibodies from blood serum of each transplant recipient were determined using flow cytometry. Peripheral blood samples from NOD mice that either rejected or accepted the islet xenografts were collected, and sera were isolated. Porcine spleen cells ($1x10^6$) obtained from the same pig islet donors were incubated with mouse serum at 1:128 dilution for 1 h at 37°C, 5% CO_2, and 95% air. Spleen cells were then washed with PBS and incubated with a 1/200 dilution of fluorescein isothiocyanate (FITC)-conjugated rat adsorbed goat anti-mouse IgG antibody (Southern Biotechnology Associates, Inc. Birmingham, Alabama, AL,

USA) for 1 h at 4°C. The percentage of cells bound to the antibody was detected from single parameter fluorescence histograms on a BD FACS Calibur flow cytometry machine (BD Biosciences Pharmingen, Mississauga, Ontario, Canada) after gating on viable lymphocytes. Controls for this experiment include sera from non-transplanted naïve non-diabetic and diabetic NOD mice, unstained porcine spleen cells, and porcine spleen cells incubated with secondary antibody alone without mouse serum.

To determine the effect of mAb therapies on the autoimmune humoral response of NOD mice similar procedure outlined above was followed except for the cells used were spleen or single islet cells from naïve non-diabetic NOD mice. These cells were incubated with 1:64 dilution of serum from NPI grafted recipients. Autoreactive anti-mouse IgG antibodies in serum were detected using a 1/100 dilution of FITC-conjugated affinity pure $F(ab)_2$ fragment rabbit anti-mouse IgG, Fcγ fragment specific antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Controls for this experiment includes sera from naïve non-diabetic and diabetic NOD mice, NOD spleen cells and NOD single islet cells which were left non-incubated or incubated with secondary antibody alone without mouse serum.

6.2.6 Detection of $CD4^+$ T cell populations in NOD mice by flow cytometery

Spleen cells ($1x10^6$) from naïve non-diabetic and diabetic NOD mice as well as NOD mice transplanted with NPI and treated with anti-CD4 mAb alone or with triple therapy were used to analyze the presence of $CD4^+$ T cell populations. Spleen cells were incubated for 30 min at 4^oC with a 1/100 dilution of

Phycoerythrin (PE)-conjugated anti-CD4 mAb (BD Pharmingen Biosciences), then washed 2 times with PBS, and suspended in 500 µl of PBS with 2% FBS. Fluorescence histograms were created using a BD FACS Calibur flow cytometry machine (BD Biosciences Pharmingen) and were used to determine the percentage of cells that expressed CD4 marker after gating on viable immune cells. Controls for this experiment include unstained or stained spleen cells with PE-conjugated anti-CD4 mAb from naive non-diabetic and diabetic NOD mice.

6.2.7 Statistical analysis

Statistical differences in graft survival and function among treated groups were determined with the Kaplan-Meier log rank test and Fisher exact test analyses, respectively using SPSS statistical software, version 11.5 for Windows (Chicago, IL, USA). A p-value <0.05 was considered to be statistically significant.

6.3 RESULTS

6.3.1 Short-term administration of a combination of anti-LFA-1 and anti-CD154 mAbs did not promote long-term survival of NPI xenografts in NOD mice.

We examined whether combined therapy of anti-LFA-1 and anti-CD154 mAbs could prevent the rejection of NPI xenografts in spontaneously diabetic NOD mice. None of the NOD mice transplanted with NPI and treated with short-term administration of anti-LFA-1 and anti-CD154 mAbs achieved normal blood glucose levels after transplantation (Figure 6.1A and Table 6.1). The majority (6/7) of recipients died between 21 and 60 days post-transplantation and the

remaining animal was sacrificed on day 22 post-transplantation due to poor health induced by diabetes.

Table 6.1: Survival of NPI xenografts in spontaneously diabetic NOD mice treated with mAbs.

Treatment	n	Graft survival (days post-transplantation)*	Number of mice that achieved normoglycemia
Short-term administration (anti-LFA-1+anti-CD154)	7	0, 0, 0, 0, 0, 0, 0	0
Double dose administration (anti-LFA-1+anti-CD154)	7	0, 0, 0, 0, 0, 0, 0	0
Long-term administration (anti-LFA-1+anti-CD154)	7	0, 0, 0, 0, 61, >100 (x2) †	3
Anti-CD4	7	0, 0, 0, 0, 0, 28, 35	2
Triple therapy (anti-LFA-1+anti-CD154 + anti-CD4)	12	0 (x3), 45, 46, 56 (x2), 59 (x2), 72, >100 (x2) ‡	9§

*Graft survival of 0 indicates that mouse did not achieve normal blood glucose level post-transplantation.
† $p=0.032$, ‡ $p<0.001$, § $p<0.003$ vs. Short-term administration group

We then assessed whether increasing the dose of anti-LFA-1 mAb could improve the survival and function of NPI in NOD mice since we previously showed that monotherapy of anti-LFA-1 but not anti-CD154 mAb prolonged NPI xenograft survival in nearly 50% of B6 mouse recipients (19). Similar to what we observed in short-term administration, all mice remained diabetic throughout the study period (Figure 6.1B and Table 6.1), and the majority (6/7) of animals died between days 10 and 46 post-transplantation due to hyperglycemia. In a parallel study, streptozotocin-induced diabetic NOD *rag-/-* mice transplanted with the

same NPI achieved normal blood glucose levels within 7 to 10 weeks post-transplantation (Figure 6.1C).

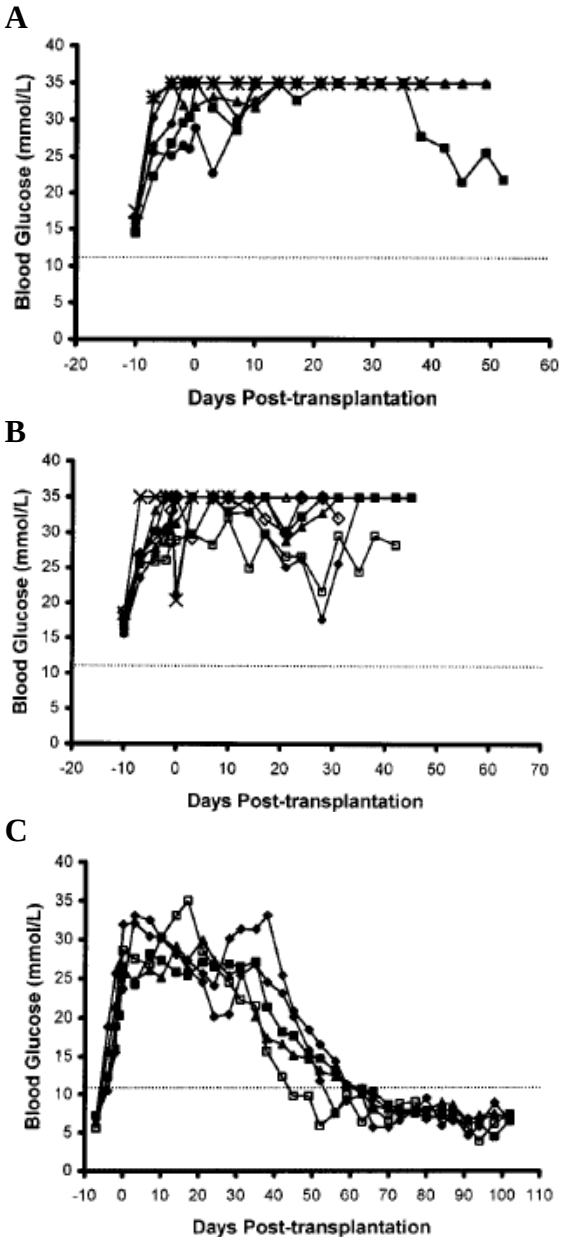

Figure 6.1: Blood glucose levels of NOD mice transplanted with NPI and treated with mAbs. NOD recipients treated with a short-term administration (A, n=7) or double dose administration (B, n=7) of anti-LFA-1 and anti-CD154 mAbs did not achieve normoglycemia post-transplantation. All streptozotocin-induced diabetic NOD *rag-/-* mice (n=5) achieved euglycemia within 7 to 10 weeks post-transplantation and maintained normoglycemia until the end of the study (>100 days, C).

Histological examination of the NPI grafts from NOD mice treated with short-term or double dose administration revealed mononuclear cellular infiltrate devoid of insulin-producing cells (Figure 6.2A and B, respectively). The majority of the cellular infiltrate were identified as $CD4^+$ T cells (Figure 6.3A and B, respectively) and macrophages (Figure 6.3C and D, respectively) with few $CD8^+$ T cells (Figure 6.3E and F, respectively) and B cells (Figure 6.3G and H, respectively). In contrast, numerous intact islets positive for insulin were present in NOD *rag-/-* mice (Figure 6.2C).

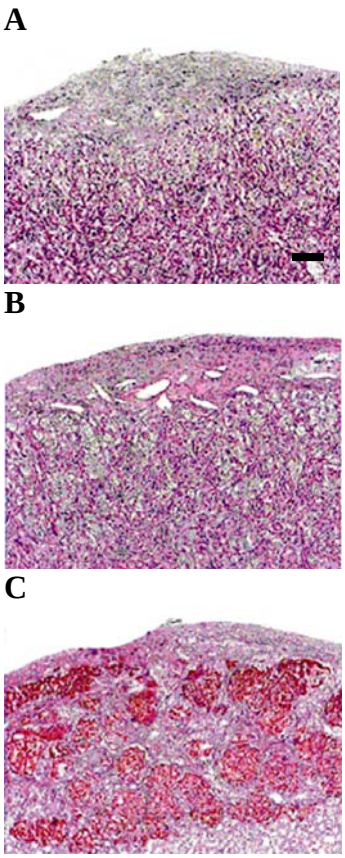

Figure 6.2: Insulin staining of NPI xenografts in NOD mice treated with short-term administration and double dose administration of a combination of anti-LFA-1 and anti-CD154 mAbs. All grafts from spontaneously diabetic NOD mice treated with short-term administration (A) and double dose administration (B) of a combination of anti-LFA-1 and anti-CD154 mAbs

consisted of immune cells with no islets staining for insulin. Intact islets with insulin-positive cells and absence of immune cell infiltrate were detected in NOD *rag-/-* mice (brown stain, C). Sections were counter stained with Harris' hematoxylin and eosin. Scale bar represents 100 µm.

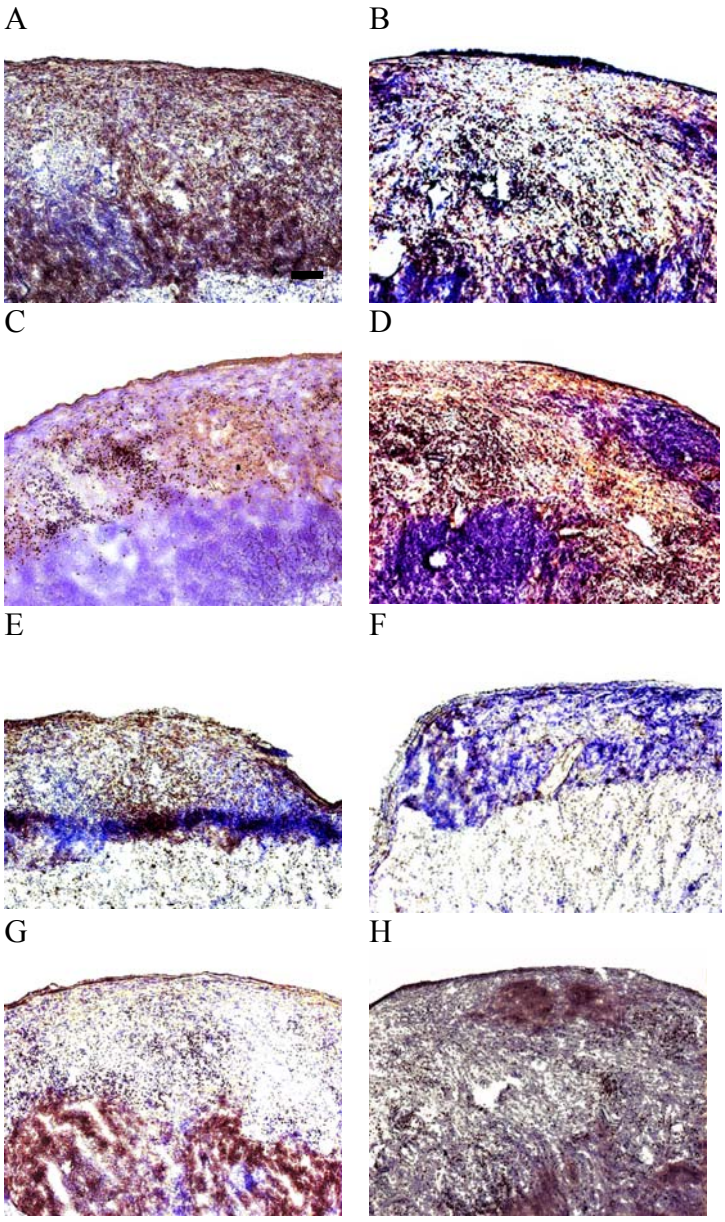

Figure 6.3: Characterization of immune cells infiltrated in the NPI xenografts in NOD mice treated with short-term administration and double dose administration of a combination of anti-LFA-1 and anti-CD154 mAbs. All grafts from spontaneously diabetic NOD mice treated with short-term

administration and double dose administration of a combination of anti-LFA-1 and anti-CD154 mAbs infiltrated with CD4 T cells (A and B, respectively), macrophages (C and D, respectively), CD8 T cells (E and F, respectively), and B cells (G and H, respectively). Scale bar represents 100 μm.

6.3.2 Long-term administration of a combination of anti-LFA-1 and anti-CD154 mAbs improved survival and function of NPI xenografts in NOD mice.

We next assessed whether long-term administration of a combination of anti-LFA-1 and anti-CD154 mAbs could improve the survival and function of NPI xenograft in NOD mice. Indeed, we found that 3/7 mice achieved normoglycemia within 5-8 weeks post-transplantation indicative of functional NPI xenografts (Figure 6.4A and Table 6.1). One mouse rejected the graft and became diabetic on day 61 and two of these mice maintained normoglycemia for more than 100 days post-transplantation. One of the two mice that had long-term graft survival reverted to diabetes on day 110 post-transplantation. NPI xenograft function was confirmed in the remaining mouse by performing a survival nephrectomy of the NPI xenograft-bearing kidney, which was followed by a rapid return to hyperglycemia. Immunohistochemical staining of NPI xenograft from a mouse with long-term surviving graft revealed some insulin-producing β cells (Figure 6.4B) and mononuclear cell infiltration, consisting of $CD4^+$ (Figure 6.4C)and $CD8^+$ T (Figure 6.4D)cells as well as B cells (Figure 6.4E) and macrophages (Figure 6.4F). These results demonstrate that long-term administration of a combination of anti-LFA-1 and anti-CD154 mAbs improved the survival and function of NPI xenografts in NOD mice compared to short-term and double dose administration of the mAbs (Table 6.1).

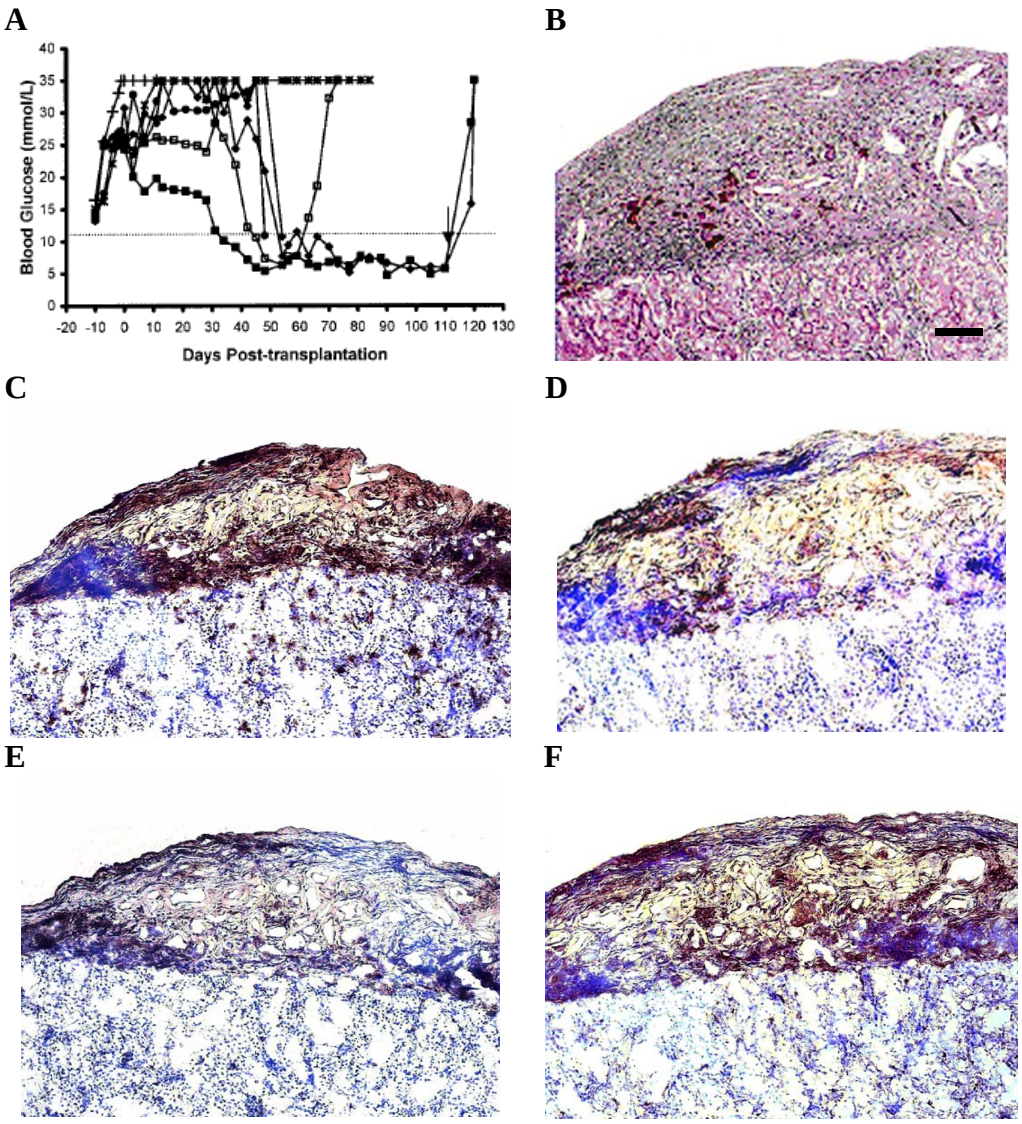

Figure 6.4: Blood glucose level and histological analysis of NPI xenografts from NOD mice treated with long-term administration of a combination of anti-LFA-1 and anti-CD154 mAbs. Three of seven mice treated with long-term administration of a combination of anti-LFA-1 and anti-CD154 mAbs achieved normoglycemia within 5-8 weeks post-transplantation (A) indicative of functional grafts. Two of these recipients maintained normoglycemia for over 100 days post-transplantation. One of these mice became diabetic on day 110 post-transplantation and graft function was confirmed in the remaining mouse by nephrectomy (arrow) of the graft-bearing kidney, which was followed by a rapid return to hyperglycemia. NPI xenografts from NOD mice treated with long-term administration of a combination of anti-LFA-1 and anti-CD154 mAbs showed some CD4 T cells (C), CD8 T cells (D), B cells (E) , and macrophages (F) infiltration with few insulin-producing cells (brown stain, B). Scale bar represents 100 μm.

6.3.3 Combination of anti-CD4 mAb with anti-LFA-1 and anti-CD154 mAbs resulted in long-term survival and function of NPI xenografts in the majority of spontaneously diabetic NOD mice.

Since $CD4^+$ T cells play an important role in the rejection of NPI xenografts (18,22,26,30) and in the pathology of autoimmune T1DM (25,31-33), we determined whether transient depletion of $CD4^+$ T cells using mAb could prevent the rejection of NPI xenografts in NOD mice. Treatment of NOD mice with depleting anti-CD4 mAb resulted in 2/7 recipients that achieved normoglycemia by 3 weeks post-transplantation (Figure 6.5A), but these mice eventually became diabetic on days 28 (which eventually died) and 35 post-transplantation indicating rejection of the NPI xenografts (Table 6.1). The majority of mice remained diabetic and two died on days 35 and 53 post-transplantation. The three remaining mice were sacrificed on days 42, 61 and 69 post-transplantation due to poor health induced by diabetes. Histological examination of the grafts showed very few insulin-producing β cells (Figure 6.5B) with intense mononuclear cell infiltration consisting of $CD4^+$ (Figure 6.5C) and $CD8^+$ T cells (Figure 6.5D) as well as B cells (Figure 6.5E), and macrophages (Figure 6.5F).

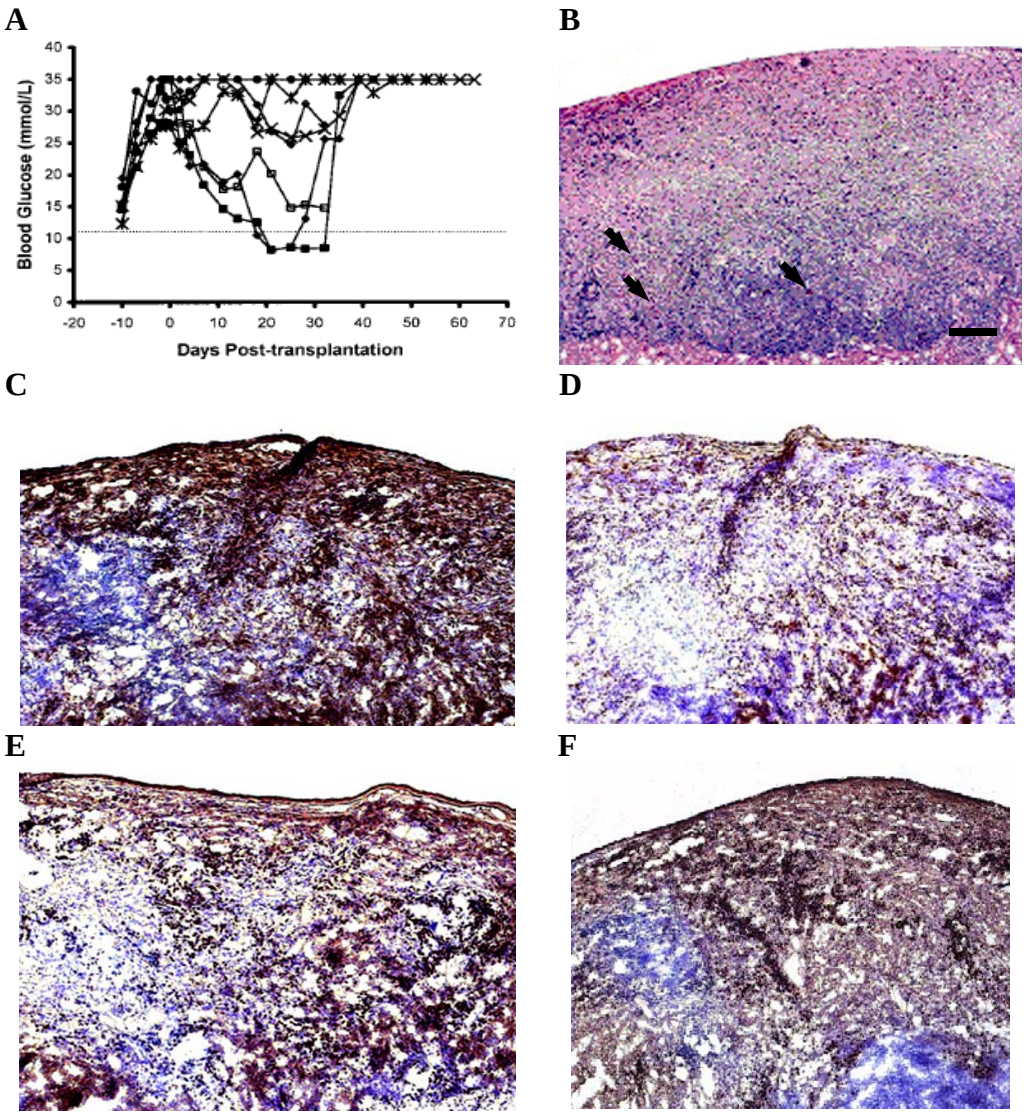

Figure 6.5: Blood glucose level and histological analysis of NPI xenografts from NOD mice treated with depleting anti-CD4 mAb. Treatment of NOD mice with depleting anti-CD4 mAb resulted in 2/7 recipients achieved normoglycemia within 3 weeks post-transplantation (A), but these mice eventually became diabetic on days 28 and 35 post-transplantation indicating rejection of the NPI xenografts. Short-term treatment of NOD mice with anti-CD4 mAb resulted in intense CD4 T cells (C), CD8 T cells (D), B cells (E), and macrophages (F) infiltration with very few insulin-positive cells (arrows, B). Scale bar represents 100 μm.

The level of $CD4^+$ T cells detected in the spleen of a mouse that had functioning graft but eventually became diabetic at 35 days post-transplantation was lower (15.86%) compared to those observed in both naïve non-diabetic (33.88% - 37.13%, n=3) and diabetic NOD mice (27.11% - 32.82%, n=3) (Figure 6.6 C, A and B, respectively).

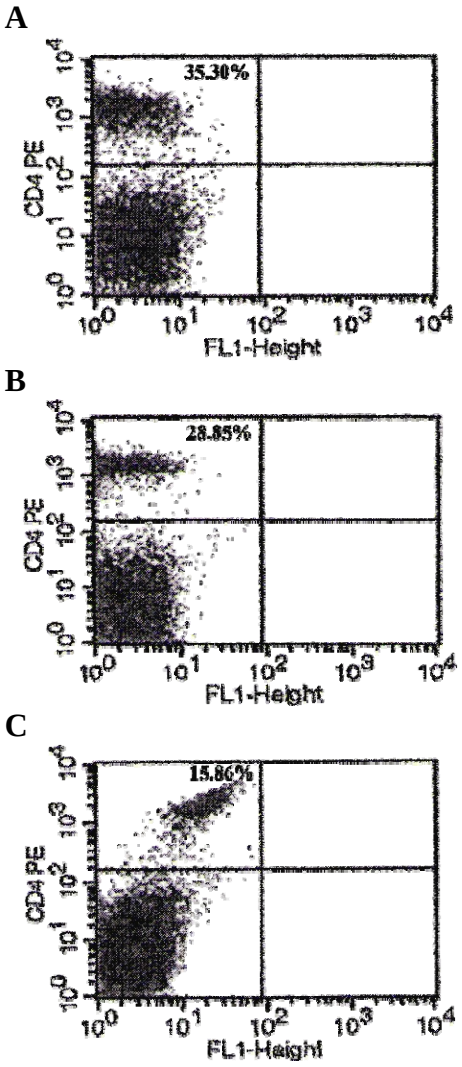

Figure 6.6: Recovery of $CD4^+$ T cells after depletion with mAb in NOD mice. The level of $CD4^+$ T cells detected in the spleen of a mouse that had functioning graft but eventually became diabetic at 35 days post-transplantation was lower (C, 15.86%) compared to those observed in both naïve non-diabetic (A, 33.88% - 37.13%, n=3) and diabetic NOD mice (B, 27.11% - 32.82%, n=3).

We then determined whether combining anti-CD4 mAb with short-term administration of anti-LFA-1 and anti-CD154 mAbs could promote long-term survival of NPI xenografts in NOD mice. Triple combination therapy of short-term administration of anti-CD4 mAb with anti-LFA-1 and anti-CD154 mAbs resulted in a significant improvement in NPI xenograft survival and function in the majority of NOD recipients compared to the other groups (Table 6.1). Nine of twelve recipients achieved normoglycemia within 5 to 8 weeks post-transplantation (Figure 6.7A). Two of these recipients maintained normoglycemia for over 100 days post-transplantation and the remaining mice became diabetic on days 42, 45, 56, 59 and 72 days post-transplantation (Table 6.1). NPI xenograft function in mice with long-term normoglycemia was confirmed by performing a survival nephrectomy of the graft-bearing kidney, which was followed by a rapid return to hyperglycemia (Figure 6.7A). Histological examination of the NPI xenografts from NOD mice with long-term islet function (>100 days) showed more insulin-producing β cells remaining in the graft compared to the other groups (Figure 6.7B). Mononuclear cell infiltration, which includes $CD4^+$ (Figure 6.7C) and $CD8^+$ T cells (Figure 6.7D) as well as B cells (Figure 6.7E) and macrophages (Figure 6.7F) was also present in the NPI xenografts examined.

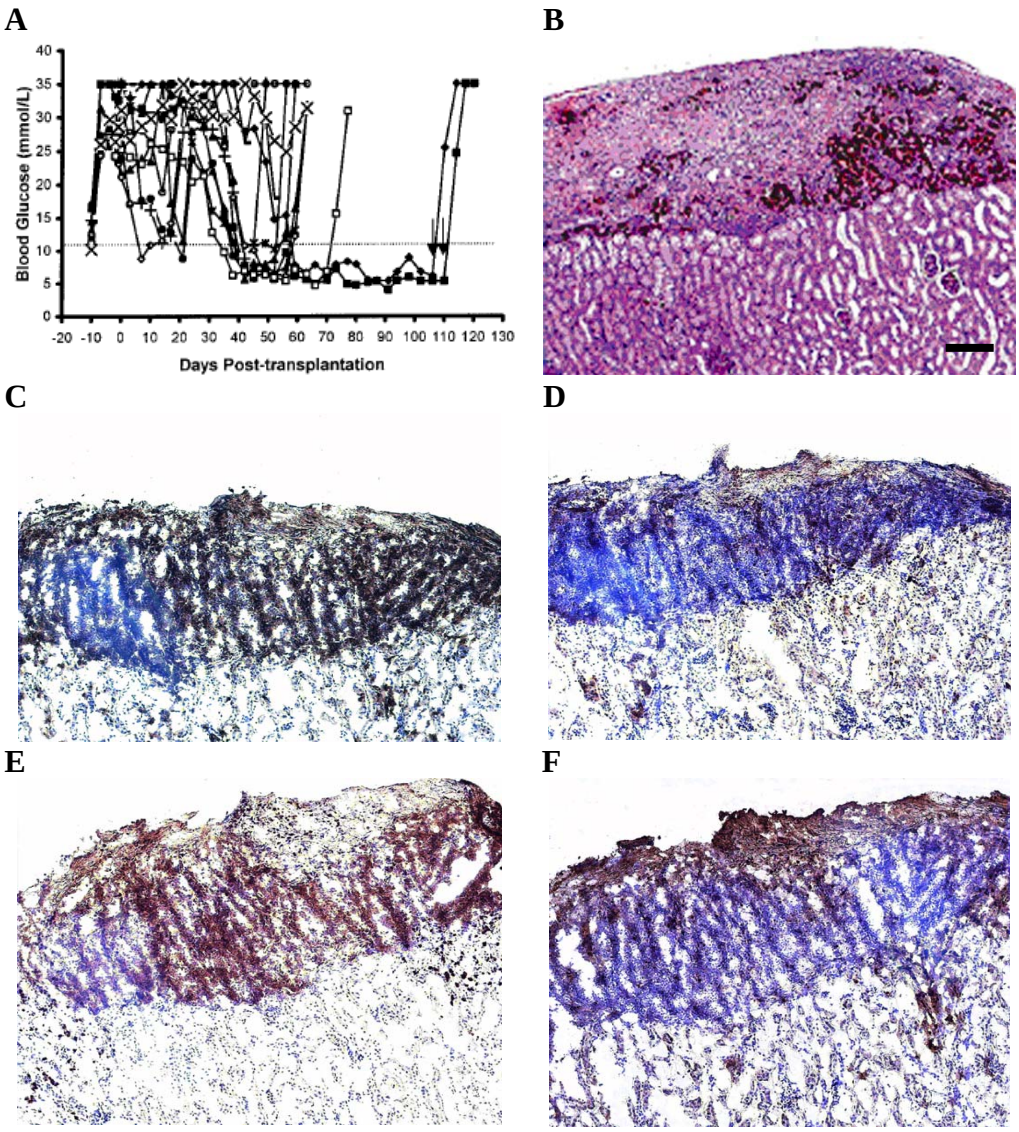

Figure 6.7: Blood glucose level and histological analysis of NPI xenografts from NOD mice treated with triple combination therapy of short-term administration of anti-CD4 mAb with anti-LFA-1 and anti-CD154 mAbs. Triple combination therapy of short-term administration of anti-CD4 mAb with anti-LFA-1 and anti-CD154 mAbs resulted 9/12 recipients achieved normoglycemia within 5 to 8 weeks post-transplantation (A). Two of these recipients maintained normoglycemia for over 100 days post-transplantation and graft function was confirmed by nephrectomy (arrows) of the graft-bearing kidney, which was followed by a rapid return to hyperglycemia. Combined triple mAb therapy resulted in fewer infiltrating CD4 T cells (C), CD8 T cells (D), B cells (E), and macrophages (F) and numerous intact islets containing insulin-positive cells (brown stain, B) present in the NPI xenografts. Scale bar represents 100 μm.

The levels of $CD4^+$ T cells in the spleen of these mice were lower (8.45% and 12.40%) (Figure 6.8A and B, respectively) compared to those observed in both naïve non-diabetic (33.88% - 37.13%, n=3) and diabetic NOD mice (27.11% - 32.82%, n=3) (Figure 6.6A and B, respectively).

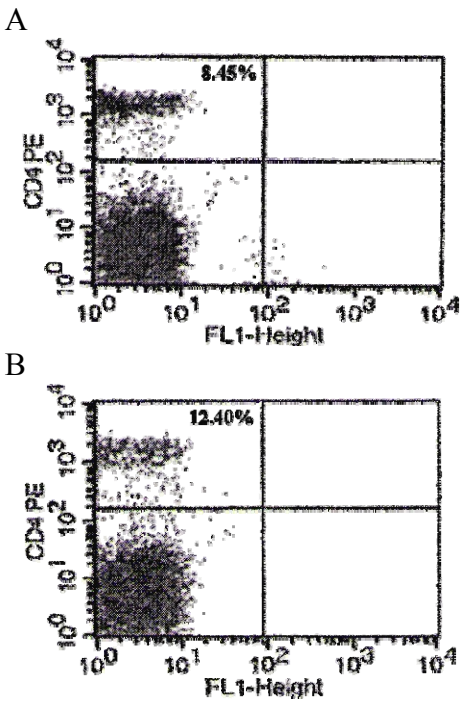

Figure 6.8: Recovery of $CD4^+$ T cells in the triple therapy treated NOD ice with long-term NPI xenograft survival. NOD mice with long-term NPI xenograft survival treated with triple therapy of anti-LFA-1, anti-CD154, and anti-CD4 mAbs displayed lower $CD4^+$ T cells (8.45%, A and 12.40%, B) compared to naïve non-diabetic and diabetic NOD mice.

Taken together, our data demonstrate that combination of anti-CD4 mAb with anti-LFA-1 and anti-CD154 mAbs significantly promotes long-term survival and function of NPI xenografts in spontaneously diabetic NOD mice.

6.3.4 Combination of anti-CD4 mAb with anti-LFA-1 and anti-CD154 mAbs inhibited the production of xenoreactive anti-porcine and autoreactive IgG antibodies in NOD mice.

Since we observed function of NPI xenografts in some of the NOD recipients treated with long-term administration of anti-LFA-1 and anti-CD154, or monotherapy of anti-CD4, or a combination of short-term administration of these monoclonal antibodies (Triple therapy), we determined the production of mouse anti-porcine antibodies in NOD recipients treated with these regimens. We found that the majority of porcine cells were bound with xenoreactive mouse IgG antibodies when porcine spleen cells from the same islet donor were incubated with sera from recipients treated with either anti-CD4 (40.29%, Figure 6.9C) or long-term administration of anti-LFA-1 and anti-CD154 mAbs (41.12%, Figure 6.9D). In contrast, mice treated with a combination of anti-CD4, anti-LFA-1 and anti-CD154 mAbs that had long-term islet xenograft survival (>100 days) displayed reduced levels of anti-porcine IgG antibodies (0.41% and 0.94%, Figure 6.9E and F), even lower than the levels found in naïve non-transplanted non-diabetic (1.32-2.44%, n=3, Figure 6.9A) and spontaneously diabetic NOD mice (1.97-4.93%, n=3, Figure 6.9B). To determine the levels of autoreactive IgG antibodies in NOD recipients treated with a combination of anti-CD4, anti- LFA-1 and anti-CD154 mAbs that had long-term islet xenograft survival, NOD spleen and islet cells were incubated with sera from these mice. The anti-spleen (0.59% and 0.76%, Figure 6.10C and D, respectively) and anti-islet (5.71% and 9.91%, Figure 6.11C and D, respectively) cell IgG antibody levels of the NOD recipients

were comparable to those detected in naïve non-diabetic NOD mice (0.55% - 1.27% and 5.93% - 9.32%, n=3 in each group, Figure 6.10A and 6.11A, respectively). These levels were much lower compared to the anti-spleen (11.90% - 44.72%, n=3, Figure 6.10B) and anti-islet (41.52% - 74.50%, n=3, Figure 6.11B) cell IgG antibodies observed in spontaneously diabetic NOD mice.

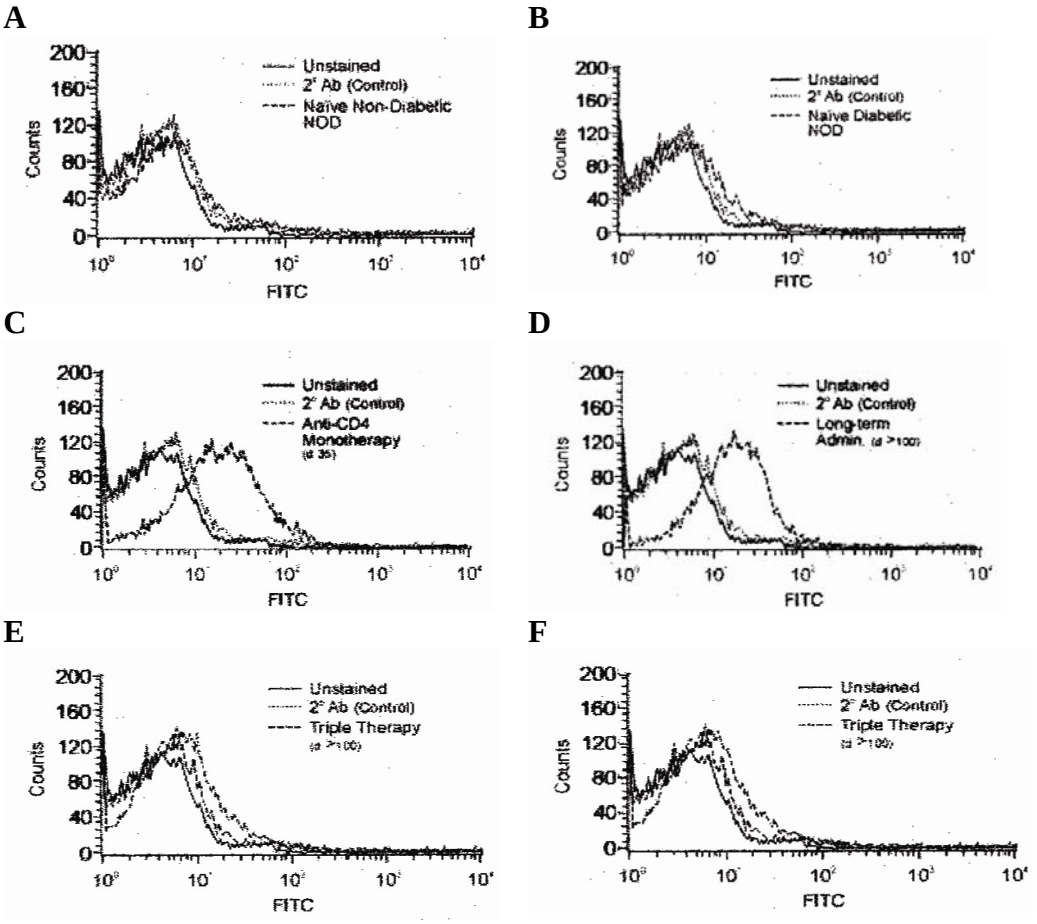

Figure 6.9: Combination of anti-CD4 mAb with anti-LFA-1 and anti-CD154 mAbs inhibited the production of mouse anti-porcine IgG antibodies in NOD mice. Neonatal porcine spleen cells (1×10^6) from islet donor pigs were incubated with 1:128 dilution of serum collected from non-transplanted naïve non-diabetic (1.50%, A) and spontaneously diabetic NOD mice (2.96%, B) or NOD mice transplanted with NPI and treated with anti-CD4 mAb (40.29%, C), long-term administration (41.12%, D), or triple therapy (0.41%, E and 0.94%, F). Representative histograms of xenoreactive IgG antibodies from control naïve non-

diabetic (1.32 - 2.44%, n=3) and spontaneously diabetic NOD mice (1.97- 4.93%, n=3) are shown. Controls for this experiment consisted of unstained cells and cells incubated with secondary antibody without serum.

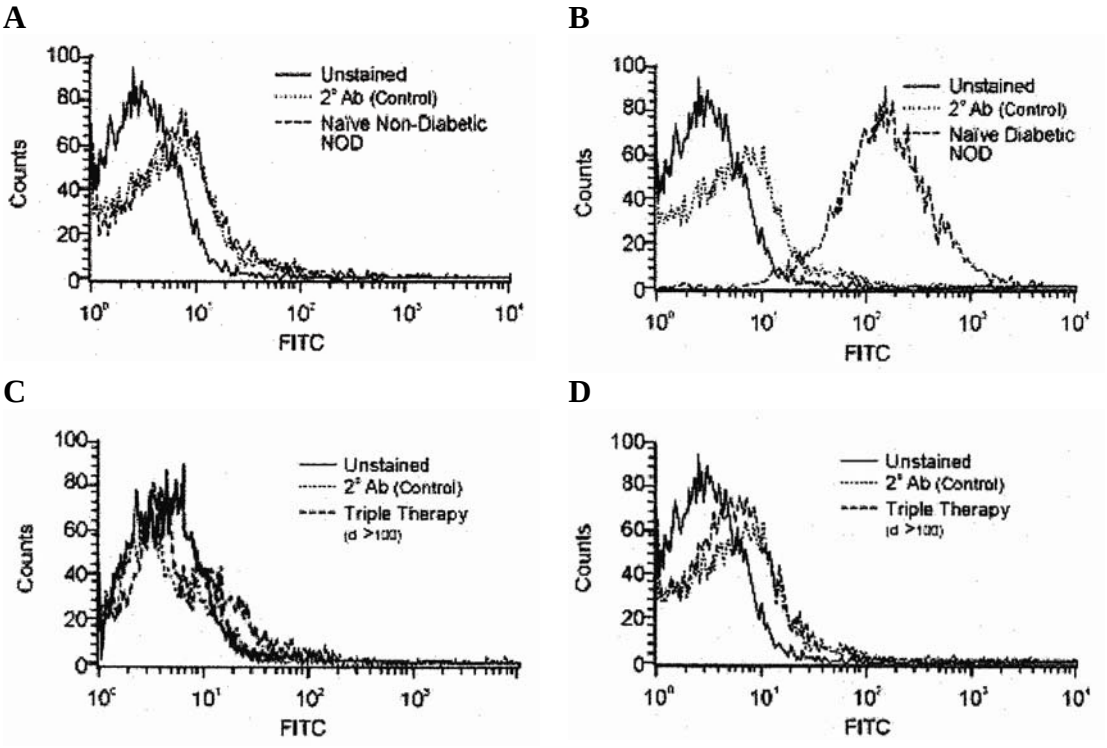

Figure 6.10: Combination of anti-CD4 mAb with anti-LFA-1 and anti-CD154 mAbs inhibited the production of autoreactive anti-spleen IgG antibodies. Naïve non-diabetic NOD spleen (1×10^6) were incubated with 1:64 dilution of serum from non-transplanted naïve non-diabetic (0.55%, A), diabetic (14.61%, B) or NOD mice treated with the triple therapy and had long-term NPI xenograft survival (0.59%, C and 0.76%, D), respectively. Shown here are representative histograms of autoreactive IgG antibodies from control naïve non-diabetic (0.55% - 1.27%, n=3, A) and spontaneously diabetic NOD mice (11.90% - 44.72%, n=3, B). Controls for this experiment consisted of unstained cells and cells incubated with secondary antibody without serum.

Taken together, these results show that the levels of xenoreactive anti-porcine and autoreactive IgG antibodies in NOD recipients treated with a combination of anti-CD4, anti-LFA-1 and anti-CD154 mAbs with long-term NPI xenograft function were inhibited.

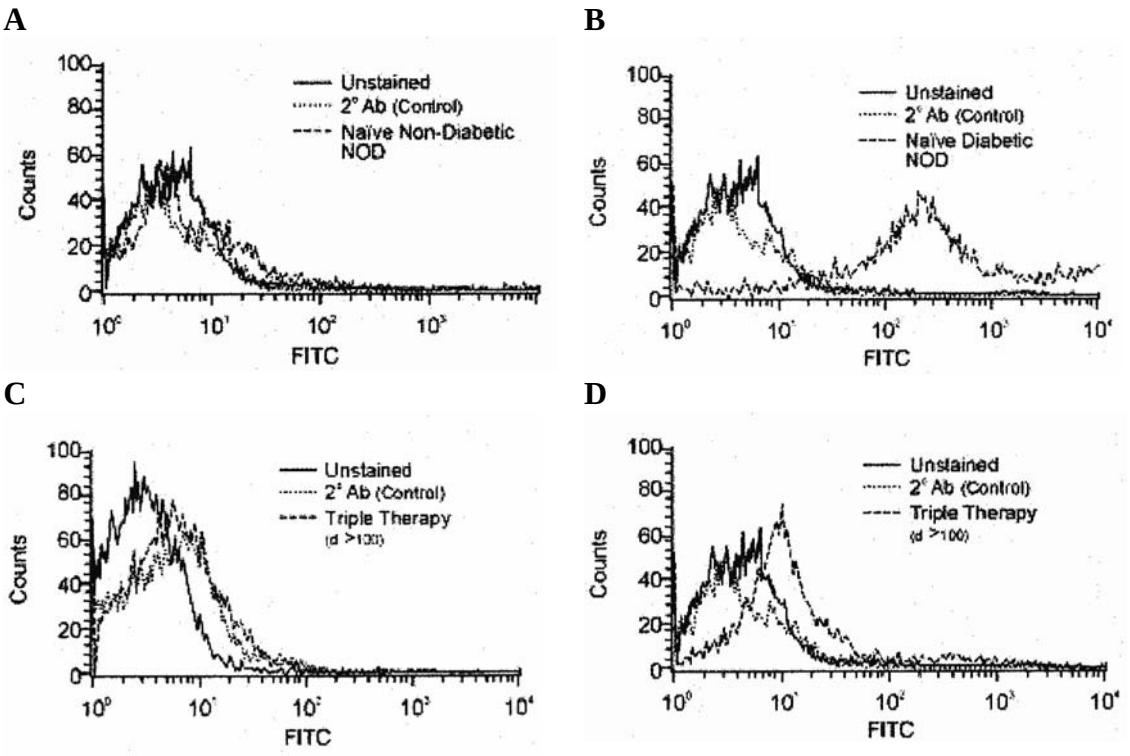

Figure 6.11: Combination of anti-CD4 mAb with anti-LFA-1 and anti-CD154 mAbs inhibited the production of autoreactive anti-islet IgG antibodies. Naïve non-diabetic NOD islet (1×10^6) cells were incubated with 1:64 dilution of serum from non-transplanted naïve non-diabetic (8.68%, A), diabetic (48.48%, B) or NOD mice treated with the triple therapy and had long-term NPI xenograft survival (5.71%, C and 9.91%, D), respectively. Shown here are representative histograms of autoreactive IgG antibodies from control naïve non-diabetic (5.93% - 9.32%, n=3, A) and spontaneously diabetic NOD mice (41.52% - 74.50%, n=3, B). Controls for this experiment consisted of unstained cells and cells incubated with secondary antibody without serum.

6.4 DISCUSSION

Islet xenotransplantation is a possible solution to the shortage of human islets for transplantation provided immune cell-mediated rejection and recurrence of T1DM can be successfully prevented. Our results show that short-term administration of a combination of anti-CD4 mAb with anti-LFA-1 anti-CD154 mAbs promotes long-term survival and function of NPI xenografts in spontaneously diabetic NOD mice. Seventy five percent (9/12) of NOD

recipients achieved normoglycemia and two mice maintained euglycemia for more than 100 days post-transplantation without additional treatment. Numerous intact islets with insulin-positive β cells were present in the graft of these mice and the levels of xenoreactive anti-porcine, and autoreactive IgG antibodies were comparable to those detected in naïve non-diabetic NOD mice.

Our results also demonstrate that short-term treatment with a combination of anti-LFA-1 and anti-CD154 mAbs which was highly effective in B6 mice (19,34) was not effective in prolonging islet xenograft survival in NOD mice even when the dose of anti-LFA-1 mAb was increased. This result was not surprising, as other studies have demonstrated that anti-rejection therapies found highly effective in promoting long-term survival of islet grafts in non-autoimmune prone mice is not always equally effective in NOD mice. The reason for this is not clear, however findings from several studies have indicated that the autoimmune disease in NOD mice might have contributed to the difficulty in achieving indefinite graft survival (35,36). It has been proposed that NOD mice with spontaneous autoimmune diabetes have a generalized defect in their response to transplantation tolerance induction (37) which could be the result of and/or controlled by the same genes that predispose the NOD mice to autoimmunity (38). Our data demonstrate that when a combination of anti-LFA-1 and anti-CD154 mAbs was administered for a longer period, modest improvement in NPI xenograft survival and but not inhibition xenoreactive antibody production were observed (Figure 6.9D). However, the majority of mice did not achieve normoglycemia suggesting

that this particular mAb regimen was not sufficient at inhibiting the xenograft rejection as well as the autoimmune responses in NOD mice.

$CD4^+$ T cells have been shown to be important in islet xenograft rejection and treatment of mice with depleting anti-CD4 mAb prolonged islet xenograft survival. However, the effect was usually transient and this has been attributed to the rapid recovery of $CD4^+$ T cells (26,28,39). In our study, single therapy of anti-CD4 mAb did not result in significant prolongation of NPI xenograft survival. Two of the seven recipients achieved normoglycemia by 3 weeks post-transplantation, but these mice rapidly returned to the diabetic state due to rejection of the islets, which was confirmed by immunohistochemical staining of the grafts. The early graft function observed in this group suggests that $CD4^+$ T cells play an important role in the early response to NPI xenografts. When anti-CD4 mAb was combined with anti-LFA-1 and anti-CD154 mAbs majority of the NOD recipients achieved normoglycemia, which was not observed in any other treatment. These results indicate that initial depletion of $CD4^+$ T cells in combination with peri-transplant administration of anti-LFA-1 and anti-CD154 mAbs can effectively inhibit the xenograft and autoimmune responses in NOD mice resulting in the delay of rejection of NPI xenografts. These results also indicate the importance of these molecules in both autoreactive and xenoreactive immune responses. The recovery of $CD4^+$ T cells may account for the rejection of NPI xenografts at later time points post-transplantation. Our study also shows that despite the presence of numerous intact islets positive for insulin in NPI xenografts collected from NOD mice treated with triple mAb therapy that had

long-term islet graft function; mononuclear cell infiltration was not completely inhibited. It will be important to determine in future studies whether the cellular infiltration will eventually destroy the entire NPI xenografts or will remain passive over time.

Taken together, our data show that significant prolongation of NPI xenograft survival can be achieved in spontaneously diabetic NOD mice using a combination of anti-CD4 mAb with anti-LFA-1 and anti-CD154 mAbs. This combination therapy inhibits both xenoreactive anti-porcine and autoreactive humoral immune responses suggesting that a collaborative interaction between $CD4^+$ T cells and B cells may have been prevented in both responses. This study also emphasizes the importance of $CD4^+$ T cells and the interaction between LFA-1 and CD154 with their ligands in the rejection of NPI xenografts in autoimmune prone NOD mice. In conclusion, blockade of T-cell costimulation and adhesion using mAbs is a promising strategy for promoting long-term survival of NPI xenografts and slight modifications of this anti-rejection protocol may result in its full potential of preventing islet xenograft rejection and disease recurrence.

6.5 REFERENCES

1. Davies,JL, Kawaguchi,Y, Bennett,ST, Copeman,JB, Cordell,HJ, Pritchard,LE, Reed,PW, Gough,SC, Jenkins,SC, Palmer,SM, .: A genome-wide search for human type 1 diabetes susceptibility genes. *Nature* 371:130-136, 1994

2. Ryan,EA, Lakey,JR, Rajotte,RV, Korbutt,GS, Kin,T, Imes,S, Rabinovitch,A, Elliott,JF, Bigam,D, Kneteman,NM, Warnock,GL, Larsen,I, Shapiro,AM: Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. *Diabetes* 50:710-719, 2001

3. Anderson,MS, Bluestone,JA: The NOD mouse: a model of immune dysregulation. *Annu.Rev.Immunol.* 23:447-485, 2005

4. Rosmalen,JG, Leenen,PJ, Pelegri,C, Drexhage,HA, Homo-Delarche,F: Islet abnormalities in the pathogenesis of autoimmune diabetes. *Trends Endocrinol.Metab* 13:209-214, 2002

5. Shapiro,AM, Lakey,JR, Ryan,EA, Korbutt,GS, Toth,E, Warnock,GL, Kneteman,NM, Rajotte,RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N.Engl.J.Med.* 343:230-238, 2000

6. Auchincloss,H, Jr., Sachs,DH: Xenogeneic transplantation. *Annu.Rev.Immunol.* 16:433-470, 1998

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

8. Hering,BJ, Wijkstrom,M, Graham,ML, Hardstedt,M, Aasheim,TC, Jie,T, Ansite,JD, Nakano,M, Cheng,J, Li,W, Moran,K, Christians,U, Finnegan,C, Mills,CD, Sutherland,DE, Bansal-Pakala,P, Murtaugh,MP, Kirchhof,N, Schuurman,HJ: Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. *Nat.Med.* 12:301-303, 2006

9. Rood,PP, Buhler,LH, Bottino,R, Trucco,M, Cooper,DK: Pig-to-nonhuman primate islet xenotransplantation: a review of current problems. *Cell Transplant.* 15:89-104, 2006

10. Yonekawa,Y, Matsumoto,S, Okitsu,T, Arata,T, Iwanaga,Y, Noguchi,H, Nagata,H, O'Neil,JJ, Tanaka,K: Effective islet isolation method with extremely high islet yields from adult pigs. *Cell Transplant.* 14:757-762, 2005

11. Korbutt,GS, Elliott,JF, Ao,Z, Smith,DK, Warnock,GL, Rajotte,RV: Large scale isolation, growth, and function of porcine neonatal islet cells. *J.Clin.Invest* 97:2119-2129, 1996

12. Rayat,GR, Rajotte,RV, Ao,Z, Korbutt,GS: 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, 2000

13. Trivedi,N, Hollister-Lock,J, Lopez-Avalos,MD, O'Neil,JJ, Keegan,M, Bonner-Weir,S, Weir,GC: Increase in beta-cell mass in transplanted porcine neonatal pancreatic cell clusters is due to proliferation of beta-cells and differentiation of duct cells. *Endocrinology* 142:2115-2122, 2001

14. Yoon,KH, Quickel,RR, Tatarkiewicz,K, Ulrich,TR, Hollister-Lock,J, Trivedi,N, Bonner-Weir,S, Weir,GC: Differentiation and expansion of beta cell mass in porcine neonatal pancreatic cell clusters transplanted into nude mice. *Cell Transplant.* 8:673-689, 1999

15. Cardona,K, Korbutt,GS, Milas,Z, Lyon,J, Cano,J, Jiang,W, Bello-Laborn,H, Hacquoil,B, Strobert,E, Gangappa,S, Weber,CJ, Pearson,TC, Rajotte,RV, Larsen,CP: Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat.Med.* 12:304-306, 2006

16. Kin,T, Korbutt,GS, Kobayashi,T, Dufour,JM, Rajotte,RV: Reversal of diabetes in pancreatectomized pigs after transplantation of neonatal porcine islets. *Diabetes* 54:1032-1039, 2005

17. Inverardi,L, Kenyon,NS, Ricordi,C: Islet transplantation: immunological perspectives. *Curr.Opin.Immunol.* 15:507-511, 2003

18. Rayat,GR, Johnson,ZA, Beilke,JN, Korbutt,GS, Rajotte,RV, Gill,RG: The degree of phylogenetic disparity of islet grafts dictates the reliance on indirect CD4 T-cell antigen recognition for rejection. *Diabetes* 52:1433-1440, 2003

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

20. Nicolls,MR, Coulombe,M, Beilke,J, Gelhaus,HC, Gill,RG: CD4-dependent generation of dominant transplantation tolerance induced by simultaneous perturbation of CD154 and LFA-1 pathways. *J.Immunol.* 169:4831-4839, 2002

21. Berney,T, Pileggi,A, Molano,RD, Poggioli,R, Zahr,E, Ricordi,C, Inverardi,L: The effect of simultaneous CD154 and LFA-1 blockade on the

survival of allogeneic islet grafts in nonobese diabetic mice. *Transplantation* 76:1669-1674, 2003

22. Guo,Z, Wu,T, Kirchhof,N, Mital,D, Williams,JW, Azuma,M, Sutherland,DE, Hering,BJ: Immunotherapy with nondepleting anti-CD4 monoclonal antibodies but not CD28 antagonists protects islet graft in spontaneously diabetic nod mice from autoimmune destruction and allogeneic and xenogeneic graft rejection. *Transplantation* 71:1656-1665, 2001

23. Lu,X, Schulz,M, Zihlmann,HR, Borel,JF: Induction of unresponsiveness to islet xenografts by a short-course treatment by anti-CD4 nondepleting monoclonal antibody. *Transplant.Proc.* 30:552-553, 1998

24. Lu,X, Schulz,M, Zihlmann,HR, Borel,JF: 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, 1998

25. Makhlouf,L, Grey,ST, Dong,V, Csizmadia,E, Arvelo,MB, Auchincloss,H, Jr., Ferran,C, Sayegh,MH: 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, 2004

26. Mandel,TE, Dillon,H, Koulmanda,M: The effect of a depleting anti-CD4 monoclonal antibody on T cells and fetal pig islet xenograft survival in various strains of mice. *Transpl.Immunol.* 3:265-272, 1995

27. Pierson,RN, III, Winn,HJ, Russell,PS, Auchincloss,H, Jr.: Xenogeneic skin graft rejection is especially dependent on CD4+ T cells. *J.Exp.Med.* 170:991-996, 1989

28. Simeonovic,CJ, Ceredig,R, Wilson,JD: Effect of GK1.5 monoclonal antibody dosage on survival of pig proislet xenografts in CD4+ T cell-depleted mice. *Transplantation* 49:849-856, 1990

29. Simeonovic,CJ, Wilson,JD, Ceredig,R: Antibody-induced rejection of pig proislet xenografts in CD4+ T cell-depleted diabetic mice. *Transplantation* 50:657-662, 1990

30. Xu,BY, Yang,H, Serreze,DV, MacIntosh,R, Yu,W, Wright,JR, Jr.: Rapid destruction of encapsulated islet xenografts by NOD mice is CD4-dependent and facilitated by B-cells: innate immunity and autoimmunity do not play significant roles. *Transplantation* 80:402-409, 2005

31. Fowell,D, Powrie,F, Saoudi,A, Seddon,B, Heath,V, Mason,D: The role of subsets of CD4+ T cells in autoimmunity. *Ciba Found.Symp.* 195:173-182, 1995

32. Hayward,AR, Shriber,M, Cooke,A, Waldmann,H: Prevention of diabetes but not insulitis in NOD mice injected with antibody to CD4. *J.Autoimmun.* 6:301-310, 1993

33. Toyoda,H, Formby,B: Contribution of T cells to the development of autoimmune diabetes in the NOD mouse model. *Bioessays* 20:750-757, 1998

34. Kobayashi,T, Harb,G, Rayat,GR: 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, 2005

35. Guo,Z, Mital,D, Shen,J, Chong,AS, Tian,Y, Foster,P, Sankary,H, McChesney,L, Jensik,SC, Williams,JW: Immunosuppression preventing concordant xenogeneic islet graft rejection is not sufficient to prevent recurrence of autoimmune diabetes in nonobese diabetic mice. *Transplantation* 65:1310-1314, 1998

36. Rivereau,AS, You,S, Lalain,S, Gouin,E, Sai,P: In vitro xenorecognition of adult pig pancreatic islet cells by splenocytes from nonobese diabetic or non-diabetes-prone mice. *Transplantation* 66:633-638, 1998

37. Markees,TG, Serreze,DV, Phillips,NE, Sorli,CH, Gordon,EJ, Shultz,LD, Noelle,RJ, Woda,BA, Greiner,DL, Mordes,JP, Rossini,AA: NOD mice have a generalized defect in their response to transplantation tolerance induction. *Diabetes* 48:967-974, 1999

38. Kishimoto,H, Sprent,J: A defect in central tolerance in NOD mice. *Nat.Immunol.* 2:1025-1031, 2001

39. Mandel,TE, Koulmanda,M, Bacelj,A: Fetal pancreas transplantation in non-obese diabetic (NOD) mice. A comparison of iso-, allo- and xenografts. *Horm.Metab Res.Suppl* 25:166-173, 1990

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1 GENERAL DISCUSSION

Autoimmune destruction of insulin producing β cells of the pancreas results in the incidence of type 1 diabetes mellitus (T1DM) which is characterized by hyperglycemia. Secondary chronic and acute complications of T1DM are associated with chronic hyperglycemia and fluctuating of blood glucose levels(1;2). Therefore, patients with T1DM require exogenous insulin to sustain their life and control the complications. 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;4). However insulin injection as a gold-standard therapy for treatment of T1DM patients, could not provide a tight degree of glycemic control as it is in the normal physiological condition in people with functional β cells (1;2). As the life expectation of patients with T1DM increased, the number of complications such as foot ulcer, retinopathy, neuropathy, and nephropathy, associated with fluctuating glucose levels increased. These complications significantly diminish the quality of life, highlighting the need for better treatment alternatives for people with T1DM (5-15). Several alternative treatment options have been known for patients with T1DM including: whole pancreas transplantation (16;17), islet transplantation (18;19), stem cell differentiation (20-23), β cell regeneration (24;25), bioartificial pancreases (26) and gene therapy (27).

Islet transplantation has recently gained worldwide attention as a viable treatment alternative for patients with T1DM. Successful islet transplantation in humans, however, did not occur until 1989 when the research team at the

University of Alberta demonstrated sustained C-peptide secretion following intraportal islet transplantation performed concurrently with orthotopic kidney transplantation (28). In 2000, the Edmonton Protocol was introduced as the first report of insulin independence in 100% of patients at 1 year following an allogeneic islet transplantation by the scientists at the University of Alberta (29). This success has thrust islet transplantation to the forefront of treatment possibilities for patients with T1DM. Despite this dramatic progress, islet transplantation at present is not a viable treatment option for the majority of people with T1DM due to the two major barriers: *i)* shortage of donor pancreatic tissue, and *ii)* the need for continuous immunosuppressive drugs to prevent the immune mediated rejection of the transplanted islet graft which have harmful side effects. These barriers have prompted investigators to look for new sources of insulin producing tissue as well as new methods of preventing the rejection of the transplanted islets which are not associated with the harmful side effects of the currently implemented immunosuppressive regimen (30;31).

To overcome the first barrier shortage of islet donors, animal (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 possibly the most ideal source for humans because of *i)* the structural similarity of porcine insulin to human insulin (32) which has been used clinically for more than 50 years (33), *ii)* the potentially unlimited supply of porcine tissue as they breed rapidly and have large litters (34), and *iii)* the potential to decrease the immunogenicity of the tissue by genetic

manipulation (35;36). Porcine islets from different age level of pig donors such as fetal, neonatal and adult have been extensively studied (37). Based on the literature only islets isolated from neonatal and adult pigs have been shown to be able to correct diabetes in large animal models as allografts (38-40) or xenografts in non-human primates (41;42).

The main challenges which should be met to make porcine islet xenotransplnatation a clinical reality included *i)* establishing a reproducible method for the efficient preparation of porcine islets, *ii)* considering the risk of transmission of porcine pathogens to humans, and *iii)* overcoming the rejection of transplanted porcine islets and ultimately strategies for induction of tolerance.

Given the fact that both neonatal and adult porcine islets have been demonstrated to function in preclinical non-human primate models (41-43), it has been suggested that these sources will serve as the donor sources for first islet xenotransplantation clinical trials. Based on current non-human primate experience using 50,000 IEQs/kg body weight (BW) of neonatal porcine islets (NPI)(41), a 70 kg human undergoing islet xenotransplantation will require 3.5 x 10^6 NPI. Given that the average 1-3 day old neonatal pig yields approximately 50,000 islet aggregates, a minimum of 70 neonatal pigs will be required for a single human NPI xenotransplant. Considering the labor-intensive nature of our current method for NPI isolation and maintained of isolated islets in culture with media change every other day for 5-7 days pre-transplantation, currently renders NPI xenotransplantation unsustainable as a treatment for T1DM. The possible ways to overcome this challenge could be decreasing the number of islets required

for transplantation by using islets from older piglets in addition to developing an automated method for isolation and media change. With regards to adult porcine islets, a human undergoing adult porcine islet xenotransplantation will require 1.75×10^6 islets (25,000 IEQs/kg BW) based on current non-human primate experience (42). A single adult pig may yield 255,000 islets, therefore an average 70 kg human recipient will require adult porcine islets from at least 6 donors which economically it is not feasible (44). Identifying a suitable strain of pigs that yields on a consistent basis more than 350,000 viable, purified, and cultured islet equivalents from a juvenile donor could be a possible way to overcome this challenge (44).

Next challenge which needs to be considered for the application of porcine islet xenotransplantation for treating large numbers of patients with T1DM, is the concern for the potential risk of zoonotic diseases, particularly viral disease as a virology research documented that pig endogenous retrovirus (PERV) can be transmitted *in vitro* from a porcine cell to a human cell-line (45). In addition, there is the potential for the transmission of infectious agents that may not normally be pathogenic in humans, but could become so in an immunocompromised patient. However none of the studies using neonatal or adult porcine islets in non-human primates (41;42) as well as fetal porcine islets in T1DM recipients (46) reported any PERV transmission in the peripheral blood or tissues harvested at necropsy. Since development of PERV-free pigs will not be practical with current technology, another strategy to minimize the risk of xenozoonosis in islet xenotransplant recipients is using the donor animals which

are designated to be pathogen-free and bred as well as maintained at a so-called “closed herd” in biosecure facilities (47). This means that in addition to the operation of facility in compliance with "Good Manufacturing Practice (GMP)", the assays for pathogens in the diagnostic laboratory need to be done in compliance with "Good Laboratory Practice (GLP)" (47). Therefore, while utilization of NPI as an islet donor source would necessitate the use of large numbers of islet donors, only breeding pairs would be maintained under GMP conditions while donor animals would be processed for islet transplantation soon after birth. On the other hand, while at least 6 adult porcine islet donors would be required for a single human islet recipient, all 6 donor animals would need to be housed and tested under GMP and GLP conditions for 10 to 36 months prior to transplantation which may escalate costs to prohibitive levels (48).

The last challenge to make porcine islet xenotransplnatation a clinical reality is overcoming the rejection of transplanted porcine islets and ultimately strategies for induction of tolerance. As it was mentioned in chapter 1 the process of porcine islet xenograft rejection comprises of IBMIR, as well as innate, antibody and cell mediated immune responses. With regards to the contribution of IBMIR in porcine islet function, it was reported that porcine islets were destroyed (22 to 73%) after 24 hours of transplantation into non-immunesuppressed non-human primate model (49). The grafts exhibited cell destruction with coagulation and complement components activation supporting that IBMIR contributed to the islet damage in this model (49). One of the possible reasons of IBMIR may be infusion of islet grafts through the intrahepatic vein which is known as only

accepted site for clinical islet transplantation (50). It was shown that using cobra venom factor to inhibit complement activation pathway and low molecular weight dextran sulfate (LMW-DS) to prevent coagulation and clotting lead to significant improvements of survival of transplanted islets in small and large animal models (51-53). In addition, development of an alternative site for islet xenotransplantation most likely will prevent or reduce IBMIR and thereby promote islet engraftment. It will also enhance the efficacy of clinically applicable immunosuppressive therapy or tolerance-induction protocols. It will provide access for histological examination of islet xenograft engraftment, rejection or acceptance, and will allow excision if infection or cancer arise within the islet xenograft (54).

Fortunately, with regards to innate and antibody dependent immune responses to porcine islet xenografts recent evidence suggests that, unlike most vascularized solid organ xenografts where hyperacute rejection result in immediate graft failure, the rejection of porcine islet xenografts in non-human primates is associated with a predominant T-cell mediated mechanism (41;42). One strategy to prevent T cell mediated rejection instead of using general immune suppressive drugs is targeting T cell activation pathways using highly specific monoclonal antibodies (mAbs) which was shown to be effective in prolonging porcine islet xenograft survival in non-human primates (41;42). It was shown by our group that short-term administrations of a combination of mAbs, particularly a combination of anti-LFA-1 and anti-CD154 mAbs is highly effective in prolonging long-term NPI xenograft survival in mouse model (43). In chapter 2 of

this manuscript it was shown that this combination can induce a stable protection of NPI xenografts for a long period time as well as induce tolerance mediated by regulatory T cells. In chapter 3 it was shown that tolerance induced to NPI xenografts by a combination of anti-LFA-1 and anti-CD154 mAbs is species-specific that it can be extended to second party porcine islet xenografts. However it can not be extended to different tissue or organ grafts (tissue-specific). The clinical application of these findings can be useful as in case of human islet transplant recipients may require a second islet transplant to maintain insulin independence. These patients may also require other organ or tissue transplantation due to the deleterious secondary tissue or organ complications. Data reported in chapter 4 show that combined anti-LFA-1 and anti-CD154 mAbs therapy can induce dominant tolerance. It was also shown that $CD4^+$ T cells play a more important role in the maintenance of tolerance and demonstrated a significant role of APC in the induction of tolerance to NPI xenografts by a combined anti-LFA-1 and nti-CD154 mAb therapy. With regards to the role of co inhibitory molecules particularly PD-1, from the data in chapter 5 it was shown that PD-1/PD-L interaction is required for the induction and maintenance of tolerance to NPI xenografts by the combined anti-LFA-1and anti-CD154 mAb therapy. Finally, in chapter 6 the efficacy of combined anti-LFA-1 and anti-CD154 mAb therapy in preventing rejection of NPI xenografts in autoimmune prone NOD mice, which is known to be an animal model of T1DM in human, was determined. Additionally, the efficacy of anti-CD4 mAb alone or in combination with anti-LFA-1 and anti-CD154 mAbs in promoting the survival of NPI

xenografts in NOD mice was examined since $CD4^+$ T cells play a pivotal role in the onset of diabetes and generation of anti-graft responses, particularly against xenografts. In this chapter it was shown that short-term administration of a combination of anti-LFA-1 with anti-CD154 plus anti-CD4 mAbs result in long-term acceptance of NPI xenografts in spontaneously diabetic NOD mice, however short-term treatment with a combination of anti-LFA-1 and anti-CD154 mAbs that was highly effective in B6 mice did not produce any significant islet xenograft prolongation in NOD mice even when the dose of anti-LFA-1 mAb was increased. A moderate improvement in the NPI xenograft survival was observed when a combination of anti-LFA-1 and anti-CD154 mAbs was administered for a longer period.

Taken together from the data reported in this thesis the combination of anti-LFA-1 and anti-CD154 mAbs can be considered a promising strategy for promoting long-term survival of NPI xenografts in mouse animal models including non-autoimmune prone B6 mouse and auto-immune prone NOD mouse models. Potential topics for future research in this subject could be: 1) elucidation the mechanism of the protective effects of this combination of mAbs, particularly looking at their effect on APC (i.e. dendritic cells and macrophages), 2) further characterization of intracellular signaling pathways modified by this combination of mAbs which could result in the induction of tolerance to NPI xenografts through the T regulatory cells.

Given the significant clinical barrier to the use of humanized anti-CD154 mAb therapy due to the thromboembolic events (55), using anti-CD40 can be

considered as an alternative strategy to block this important costimulation pathway to prevent graft rejection (56-59). Considering the availability of humanized form of anti-LFA-1 (CD11a), Efalizumab, and potential application of anti-CD40 mAb, targeting these pathways may result in its full potential of preventing islet xenograft rejection in clinical porcine islet xenotransplnation.

7.2 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, 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. Porcine islets are a readily available source of donor tissue which can meet the demands of islet transplantation. However, careful control of porcine donor source tissue will be essential. In this thesis we have shown that short-term administrations of anti-LFA-1 and anti-CD154 mAbs can successfully prolong NPI xenograft survival indefinitely and induce dominant, species- and tissue-specific tolerance mediated by regulatory T cells in non-autoimmune prone B6 mice. It was also shown that this combination of mAbs is significantly effective in promoting NPI xenografts survival in autoimmune prone NOD mice when combined with anti-CD4 mAb. It is may hope that the research presented in this thesis will provide insight into the nature of the immune responses 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.

7.3 REFERENCES

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

2. Kasper D AFDLESHaJJ, ed.Harrison's Principles of InternalMedicine - 16th Ed.USA: McGraw-Hill Companies: Harrison's Principles of Internal Medicine. New York, Knopf: Distributed by Random House, 2005,

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

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

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

6. Tripathi,BK, Srivastava,AK: Diabetes mellitus: Complications and therapeutics. *Med.Sci.Monit.* 12:RA130-RA147, 2006

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

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

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

10. Haller,MJ, Atkinson,MA, Schatz,D: Type 1 diabetes mellitus: etiology, presentation, and management. *Pediatr.Clin.North Am.* 52:1553-1578, 2005

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

12. Shore,AC: The microvasculature in type 1 diabetes. *Semin.Vasc.Med.* 2:9-20, 2002

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

14. Libby,P, Nathan,DM, Abraham,K, Brunzell,JD, Fradkin,JE, Haffner,SM, Hsueh,W, Rewers,M, Roberts,BT, Savage,PJ, Skarlatos,S, Wassef,M, Rabadan-Diehl,C: 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, 2005

15. Dahl-Jorgensen,K, Larsen,JR, Hanssen,KF: Atherosclerosis in childhood and adolescent type 1 diabetes: early disease, early treatment? *Diabetologia* 48:1445-1453, 2005

16. Cohen,DJ, St Martin,L, Christensen,LL, Bloom,RD, Sung,RS: Kidney and pancreas transplantation in the United States, 1995-2004. *Am.J.Transplant.* 6:1153-1169, 2006

17. Humar,A, Kandaswamy,R, Granger,D, Gruessner,RW, Gruessner,AC, Sutherland,DE: Decreased surgical risks of pancreas transplantation in the modern era. *Ann.Surg.* 231:269-275, 2000

18. Ryan,EA, Paty,BW, Senior,PA, Bigam,D, Alfadhli,E, Kneteman,NM, Lakey,JR, Shapiro,AM: Five-year follow-up after clinical islet transplantation. *Diabetes* 54:2060-2069, 2005

19. Shapiro,AM, Lakey,JR, Paty,BW, Senior,PA, Bigam,DL, Ryan,EA: Strategic opportunities in clinical islet transplantation. *Transplantation* 79:1304-1307, 2005

20. Meier,JJ, Bhushan,A, Butler,PC: The potential for stem cell therapy in diabetes. *Pediatr.Res.* 59:65R-73R, 2006

21. Madsen,OD: Stem cells and diabetes treatment. *APMIS* 113:858-875, 2005

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

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

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

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

26. Jaremko,J, Rorstad,O: Advances toward the implantable artificial pancreas for treatment of diabetes. *Diabetes Care* 21:444-450, 1998

27. Yoon,JW, Jun,HS: Recent advances in insulin gene therapy for type 1 diabetes. *Trends Mol.Med.* 8:62-68, 2002

28. Warnock,GL, Kneteman,NM, Ryan,EA, Evans,MG, Seelis,RE, Halloran,PF, Rabinovitch,A, Rajotte,RV: Continued function of pancreatic islets after transplantation in type I diabetes. *Lancet* 2:570-572, 1989

29. Shapiro,AM, Lakey,JR, Ryan,EA, Korbutt,GS, Toth,E, Warnock,GL, Kneteman,NM, Rajotte,RV: Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N.Engl.J.Med.* 343:230-238, 2000

30. Jonathan,RT, Mohammadreza,M, Shapiro,AM: Current status of clinical islet cell transplantation. *Methods Mol.Biol.* 333:47-104, 2006

31. Merani,S, Shapiro,AM: Current status of pancreatic islet transplantation. *Clin.Sci.(Lond)* 110:611-625, 2006

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

33. Rother,KI, Harlan,DM: Challenges facing islet transplantation for the treatment of type 1 diabetes mellitus. *J.Clin.Invest* 114:877-883, 2004

34. Korbutt,GS, Elliott,JF, Ao,Z, Smith,DK, Warnock,GL, Rajotte,RV: Large scale isolation, growth, and function of porcine neonatal islet cells. *J.Clin.Invest* 97:2119-2129, 1996

35. Phelps,CJ, Koike,C, Vaught,TD, Boone,J, Wells,KD, Chen,SH, Ball,S, Specht,SM, Polejaeva,IA, Monahan,JA, Jobst,PM, Sharma,SB, Lamborn,AE, Garst,AS, Moore,M, Demetris,AJ, Rudert,WA, Bottino,R, Bertera,S, Trucco,M, Starzl,TE, Dai,Y, Ayares,DL: Production of alpha 1,3-galactosyltransferase-deficient pigs. *Science* 299:411-414, 2003

36. Dor,FJ, Tseng,YL, Cheng,J, Moran,K, Sanderson,TM, Lancos,CJ, Shimizu,A, Yamada,K, Awwad,M, Sachs,DH, Hawley,RJ, Schuurman,HJ, Cooper,DK: alpha1,3-Galactosyltransferase gene-knockout miniature swine produce natural cytotoxic anti-Gal antibodies. *Transplantation* 78:15-20, 2004

37. Korbutt,GS: What type of islets should be used? *Xenotransplantation* 15:81-82, 2008

38. Kin,T, Korbutt,GS, Kobayashi,T, Dufour,JM, Rajotte,RV: Reversal of diabetes in pancreatectomized pigs after transplantation of neonatal porcine islets. *Diabetes* 54:1032-1039, 2005

39. Brandhorst,D, Brandhorst,H, Hering,BJ, Bretzel,RG: Long-term survival, morphology and in vitro function of isolated pig islets under different culture conditions. *Transplantation* 67:1533-1541, 1999

40. Mellert,J, Hering,BJ, Liu,X, Brandhorst,D, Brandhorst,H, Federlin,K, Bretzel,RG, Hopt,UT: Intravenous glucose tolerance tests after porcine islet auto- and allotransplantation. *Transplant.Proc.* 29:2091-2092, 1997

41. Cardona,K, Korbutt,GS, Milas,Z, Lyon,J, Cano,J, Jiang,W, Bello-Laborn,H, Hacquoil,B, Strobert,E, Gangappa,S, Weber,CJ, Pearson,TC, Rajotte,RV, Larsen,CP: Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. *Nat.Med.* 12:304-306, 2006

42. Hering,BJ, Wijkstrom,M, Graham,ML, Hardstedt,M, Aasheim,TC, Jie,T, Ansite,JD, Nakano,M, Cheng,J, Li,W, Moran,K, Christians,U, Finnegan,C, Mills,CD, Sutherland,DE, Bansal-Pakala,P, Murtaugh,MP, Kirchhof,N, Schuurman,HJ: Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. *Nat.Med.* 12:301-303, 2006

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

44. Prabhakaran,S, Hering,BJ: What strain of pig should be used? *Xenotransplantation* 15:83-86, 2008

45. Patience,C, Takeuchi,Y, Weiss,RA: Infection of human cells by an endogenous retrovirus of pigs. *Nat.Med.* 3:282-286, 1997

46. Heneine,W, Tibell,A, Switzer,WM, Sandstrom,P, Rosales,GV, Mathews,A, Korsgren,O, Chapman,LE, Folks,TM, Groth,CG: No evidence of infection with porcine endogenous retrovirus in recipients of porcine islet-cell xenografts. *Lancet* 352:695-699, 1998

47. Schuurman,HJ: Regulatory aspects of pig-to-human islet transplantation. *Xenotransplantation* 15:116-120, 2008

48. O'Connell,PJ: The rationale and practical issues for the maintenance of clean herds for clinical islet xenotransplantation. *Xenotransplantation* 15:91-92, 2008

49. Kirchhof,N, Shibata,S, Wijkstrom,M, Kulick,DM, Salerno,CT, Clemmings,SM, Heremans,Y, Galili,U, Sutherland,DE, Dalmasso,AP, Hering,BJ: Reversal of diabetes in non-immunosuppressed rhesus macaques by intraportal porcine islet xenografts precedes acute cellular rejection. *Xenotransplantation* 11:396-407, 2004

50. Moberg,L, Johansson,H, Lukinius,A, Berne,C, Foss,A, Kallen,R, Ostraat,O, Salmela,K, Tibell,A, Tufveson,G, Elgue,G, Nilsson,EK, Korsgren,O, Nilsson,B: Production of tissue factor by pancreatic islet cells as a trigger of detrimental thrombotic reactions in clinical islet transplantation. *Lancet* 360:2039-2045, 2002

51. Rood,PP, Bottino,R, Balamurugan,AN, Smetanka,C, Ayares,D, Groth,CG, Murase,N, Cooper,DK, Trucco,M: Reduction of early graft loss after

intraportal porcine islet transplantation in monkeys. *Transplantation* 83:202-210, 2007

52. Goto,M, Groth,CG, Nilsson,B, Korsgren,O: 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, 2004

53. Goto,M, Johansson,H, Maeda,A, Elgue,G, Korsgren,O, Nilsson,B: Low molecular weight dextran sulfate prevents the instant blood-mediated inflammatory reaction induced by adult porcine islets. *Transplantation* 77:741-747, 2004

54. Contreras,JL: Extrahepatic transplant sites for islet xenotransplantation. *Xenotransplantation* 15:99-101, 2008

55. Kawai,T, Andrews,D, Colvin,RB, Sachs,DH, Cosimi,AB: Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat.Med.* 6:114, 2000

56. Aoyagi,T, Yamashita,K, Suzuki,T, Uno,M, Goto,R, Taniguchi,M, Shimamura,T, Takahashi,N, Miura,T, Okimura,K, Itoh,T, Shimizu,A, Furukawa,H, Todo,S: A Human Anti-CD40 Monoclonal Antibody, 4D11, for Kidney Transplantation in Cynomolgus Monkeys: Induction and Maintenance Therapy. *Am.J.Transplant.* 2009

57. Adams,AB, Shirasugi,N, Jones,TR, Durham,MM, Strobert,EA, Cowan,S, Rees,P, Hendrix,R, Price,K, Kenyon,NS, Hagerty,D, Townsend,R, Hollenbaugh,D, Pearson,TC, Larsen,CP: Development of a chimeric anti-CD40 monoclonal antibody that synergizes with LEA29Y to prolong islet allograft survival. *J.Immunol.* 174:542-550, 2005

58. Pearson,TC, Trambley,J, Odom,K, Anderson,DC, Cowan,S, Bray,R, Lin,A, Hollenbaugh,D, Aruffo,A, Siadak,AW, Strobert,E, Hennigar,R, Larsen,CP: Anti-CD40 therapy extends renal allograft survival in rhesus macaques. *Transplantation* 74:933-940, 2002

59. Gangappa,S, Larsen,CP: Immunosuppressive protocols for pig-to-human islet transplantation: lessons from pre-clinical non-human primate models. *Xenotransplantation* 15:107-111, 2008