ABSTRACT

Analysis of Inflammatory Changes in Human Pancreatic Islet Cells

Andrew M. Jackson, Ph.D.

Mentor: Bashoo Naziruddin, Ph.D.

Pancreatic islet cell transplantation is a promising investigational research treatment for labile type 1 diabetes mellitus. However, several obstacles still exist to the implementation of clinical islet cell transplantation as a standard therapy. These obstacles include a shortage of donor pancreata, an imperfect islet isolation procedure, significant loss of islets during the peri-transplant period, and islet toxicity of immunosuppressive drugs. The present study is focused on addressing two of these major hurdles; namely, identification of factors affecting the islet isolation, and understanding the mechanism involved in the peri-transplant loss of islets.

Initial analysis was centered on the effect of cold ischemia time (CIT) on the islet isolation outcome.

Comparison of varying CIT and several factors that determine the quantity and quality of islets obtained from 52 isolations was performed. This analysis showed that CIT of less than four hours significantly improved islet isolation results. This finding could help improve the current strategy used in clinical islet transplantation.

We hypothesized that following transplantation, the inflammatory environment will specifically alter gene expression in transplanted islets, and also will induce surface expression of HLA-class II molecules. This, in turn, could cause anti-donor response resulting in islet destruction.

To test the above hypotheses, two studies were performed. Islet cells were treated with control or type 1 diabetic serum. Gene expression was then analyzed using micro array and confirmed by real-time PCR. Islets treated with diabetic serum demonstrated specific induction of multiple genes reported to have secondary roles in angiogenesis while inhibiting transcription of genes with protective attributes against environmental stresses.

Islet cells were treated with IFNγ and TNFα and analyzed for HLA class II induction by real-time PCR analysis, flow cytometry, and immunofluorescent imaging. Cytokine treated islets demonstrated significantly

upregulated HLA class II gene transcription and surface expression. Importantly, islet transplant recipient serum showed increased binding and cytotoxicity specifically directed against cytokine treated islets.

Together these data suggest that in the context of hepatic portal vein transplantation, islet cells contribute to the innate and adaptive immune response during the peri-transplant period resulting in islet destruction.

Analysis of Inflammatory Changes in Human Pancreatic Islet Cells

by

Andrew M. Jackson, B.S., M.S.

A Dissertation

Approved by the Department of Biomedical Studies

_____
Robert R. Kane, Ph.D., Chairperson

Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of
Doctor of Philosophy

Approved by the Dissertation Committee

_____
Bashoo Naziruddin, Ph.D., Chairperson

_____
Shinichi Matusmoto, M.D., Ph.D.

_____
Joseph T. Newman, Ph.D.

_____
Christopher M. Kearney, Ph.D.

_____
William D. Hillis, M.D.

Accepted by the Graduate School
May 2009

_____
J. Larry Lyon, Ph.D., Dean

TABLE OF CONTENTS

	Page
LIST OF FIGURES	viii
LIST OF TABLES	x
LIST OF ABBREVIATIONS	xi
ACKNOWLEDGMENTS	xv
DEDICATION	xvii
CHAPTER ONE	1
Introduction	1
Diabetes and Transplantation	3
History	3
Etiology	10
Pancreas Transplantation	12
Early Islet Transplantation	13
A New Beginning	14
The Edmonton Protocol	14
Baylor Islet Cell Program	16
Challenges Facing Islet Transplant Centers	17
Shortage of Donors	17
Efficiency of Isolation	18
Islet Quality Prior to Transplant	18
Improving Islet Engraftment	19

Overcoming the Immune Response 20

Current Status of Islet Transplantation 21

Multiple Donors Still Required to Attain Insulin Independence 21

Donor Specific Sensitization 22

Focus of Dissertation 22

Cold Ischemic Time and Islet Cell Isolation 23

Islet Cell Response to T1DM Auto-Antibodies and Complement 24

HLA Expression on Isolated Pancreatic Islet Cells 25

CHAPTER TWO 26

Prolonged Cold Ischemia has a Detrimental Effect on the Outcome of Islet Cell Isolation 26

Introduction 26

Materials and Methods 32

Procurement of Pancreata 32

Islet Cell Isolation 34

Islet Cell Assessment 35

Data Collection 36

Statistical Methods 37

Results 37

Prolonged Cold Ischemic Time Reduces the Quantity of Isolated Islet Cells 37

Prolonged Cold Ischemic Time Influences the Effectiveness of Enzyme 40

Prolonged Cold Ischemia Adversely Affects the Quality of Isolated Islets 43

Discussion 44

CHAPTER THREE 48

The Role of Complement in the Destruction of Islet Grafts Post-Transplant 48

Introduction 48

Materials and Methods 50

Pancreas Procurement and Islet Isolation 50

Serum Treatment of Islet Cells 51

Immunofluorescent Imaging 52

Microarray Analysis 54

Real Time PCR 56

Statistical Methods 56

Results 57

Complement Activation by Treatment of Islets with Type 1 Diabetic Serum 57

Complement Induces Gene Expression Changes in Isolated Islets 60

Real-time QPCR of Select Genes Corroborates Changes Indicated by Micro-Array Analysis 63

Discussion 66

CHAPTER FOUR 74

The Expression of HLA Class II Molecules on the Surface of Isolated Islet Cells and their Role in Sensitization and Rejection 74

Introduction 74

Materials and Methods 80

Pancreas Procurement and Islet Isolation 80

Cytokine Treatment 81

Real-Time PCR 82

Immunofluorescent Imaging 83

Flow Cytometry 85

Examination for Binding of Anti-HLA-DR7 Positive Serum from Islet Transplant Recipient to Cytokine Treated DR7+ Islets 86

Complement Mediated Cytotoxicity Assay 87

Statistical Methods 88

Results 89

IFNγ and TNFα Treatment of Isolated Human Pancreatic Islets Results in Up-Regulation of CIITA, HLA-DRα, and HLA-DRβ1 Transcripts 89

HLA class II Expression on Human Islets Treated With Cytokines 91

Islet Transplant Recipient Serum Positive for Anti-HLA-DR7 Binds to Cytokine Treated DR7+ Islets 94

Donor Specific Antibodies Against HLA-DR7 Induce Complement Mediated Cytotoxicity in Islet Cells Treated with IFN-γ and TNF-α 94

Discussion 97

CHAPTER FIVE 104

Summary and Conclusions 104

Summary 104

Prolonged Cold Ischemia has a Detrimental Effect on the Outcome of Islet Cell Isolation 104

The Role of Complement in the Destruction of Islet Grafts Post-Transplant 106

The Expression of HLA Class II Molecules on the Surface of Isolated Islet Cells and their Role in Sensitization and Rejection 107

Conclusions 109

APPENDIX 112

APPENDIX 113

Author Permissions 113

BIBLIOGRAPHY 126

LIST OF FIGURES

Figure		Page
1.1	Structure of the Human Pancreas	7
1.2	Architecture of Islet Cells Differ Between Species	9
2.1	Pancreas Cleaning, Perfusion, and Digestion	27
2.2	Islet Cell Counting and Viability Assessment	29
2.3	Prolonged Cold Ischemia Adversely Influences Islet Cell Liberation and Purification	39
2.4	The Length of CIT and the use of Enzyme Significantly Affects the Islet Cell Liberation Yield	42
3.1	Binding of Auto-Antibodies From Diabetic Serum to Islet Cells.	58
3.2	Complement Deposited on Islets Exposed to Type 1 Diabetic Serum.	59
3.3	Complement Activation Initiated by Autoantibodies in Type 1 Diabetic Serum Induce a Specific Profile of Gene Expression.	61
3.4	Class Prediction Analysis of Islets Exposed to Diabetic Serum Based on Changes in Genetic Profile	62
3.5	Real Time PCR Analysis Confirms Micro-Array Data in Representative Genes	66
4.1	Structure of the Human Leukocyte Antigen (HLA)	77
4.2	Direct and Indirect Pathways of Antigen Recognition	78

4.3 Induction of class II human leukocyte antigen genes in isolated human islet cells by treatment with IFNγ and TNFα 90

4.4 Islet cells express HLA-DR7 on their surface following treatment with TNFα and IFNγ 92

4.5 Upregulation of HLA-DR Expressed on Islet Cells 93

4.6 Islet transplant recipient anti-HLA-DR7 binds to HLA-DR7+ islet cells 95

4.7 Treatment with IFN-γ and TNF-α Leads to Increased Complement Mediated Cytotoxicity 96

LIST OF TABLES

Table	Page
1.1 The Islet Content of Human Pancreas	9
2.1 Donor Characteristics	32
2.2 One way ANOVA analysis of Isolation Data	38
2.3 Two way ANOVA Analysis of Isolation Data	41
2.4 One way ANOVA Analysis of Islet Cell Quality	43
3.1 Average Signal Intensity for Class Predictor Genes	64
4.1 HLA Sensitization in Islet Transplant Recipients	76
4.2 HLA Characterization of Donor Islets	81
4.3 Quality of Islet Cell Preparations	82

LIST OF ABBREVIATIONS

AF	Alexa Fluor
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
ATG	Anti Thymocyte Globulin
BICT	Baylor Islet Cell Transplant
BMI	Body Mass Index
BRTI	Baylor Regional Transplant Institute
BSA	Bovine Serum Albumin
cDNA	Complimentary DNA
CIITA	Class Two Trans Activator
CIT	Cold Ischemic Time
cRNA	Complimentary RNA
CTA	Cell Transplant Association
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
DAPI	4’,6-diamidino-2-phenylindole
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DRI	Diabetes Research Institute
EBI	Eppstein Barr Inducible
ELISA	Enzyme Linked Immune Sandwich Assay
FACS	Fluorescence Assisted Cell Sorting

FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FDA	Fluorescein Diacetate
FITC	Fluorescein Isothiocyanate
GAD	Glutamic Acid Decaroxylase
GAPDH	Glyceraldehye 3-Phophate DeHydrogenase
GOI	Gene of Interest
GTP	Guanosine Triphosphate
HLA	Human Leukocyte Antigen
HAS	Human Serum Albumin
IA2A	Insulin Antigen 2A (tyrosine phosphatase related)
IAA	Insulin Auto-Antibody
IBMIR	Instant Blood-Mediated Inflammatory Reaction
ICA	Islet Cell Antigen
IEQ	Islet Equivalent
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin Growth Factor
IL	Interleukin
IND	Investigational Drug Application
IPITA	International Pancreas and Islet Transplant Association
IXA	International Xenotransplantation Association
MHC	Major Histocompatibility Complex

MIN6	Mouse Insulinoma
M-Kyoto	Modified Kyoto
MMP	Matrix Metalloproteinase
mRNA	Messenger RNA
MTF	Mammalian Tissue Free
MT	Metalothionene
$NF_{\kappa}B$	Nuclear Factor Kappa B
NK	Natural Killer
OCT	Optimal Cutting Temperature
OPO	Organ Procurement Organization
PAK	Pancreas After Kidney
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerytherin
PFA	Paraformaldehyde
PFC	Perfluorocarbon
PI	Propidium Idodide
PRA	Panel Reactive Antibody
PTA	Pancreas Transplant Alone
QPCR	Quantitative PCR
RAD	Ras Associated with Diabetes
RN	Recteptor Antagonist
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species

SEM	Standard Error of the Mean
SPK	Simultaneous Pancreas Kidney
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TGF	Transforming Growth Factor
Th	T Helper Lymphocte
TIMP	Tissue Inhibitor of Metalloproteinase
TLM	Two Layer Method
TNF	Tumor Necrosis Factor
UV	Ultraviolet
UW	University of Wisconsin
ZnT	Zinc Transporter
β-ME	Beta Mercaptoethanol

ACKNOWLEDGMENTS

I am very fortunate to have had an excellent doctoral committee and I would like to extend my gratitude to Drs. Shinichi Matsumoto, Joseph Newman, Christopher Kearney, and William Hillis. I especially would like to thank Dr. William Hillis for sparking my interest in immunology. I am very grateful to Dr. John E. Connolly, who took much time out of his busy schedule to train me in microscopy and immunofluorescence. I would also like to extend my sincere gratitude to Dr. Damien Chaussabel and his microarray core for all of their training, support, and use of their equipment. I would like to thank Drs. Tetsuya Ikemoto, Masayuki Shimoda, and Hirofumi Noguchi for training me in surgical techniques. I would like to thank the Institute of Biomedical Studies for the support of my doctoral work. To my mentor, Dr. Bashoo Naziruddin, I sincerely thank him for training me to be a scientist. I thank him for his support, patience, guidance, and encouragement in the development of my career. I would like to thank my parents for allowing me to study abroad, and for all of their support. I would like to thank my beautiful wife, Sarah, for all of her support, patience, understanding,

encouragement, and love. Finally, I would like to thank my Lord and Savior Jesus Christ for His guidance and provision on this journey He has lead me on, and without whom none of this would have been possible.

DEDICATION

To my loving wife and family

CHAPTER ONE

Introduction

Today Diabetes Mellitus is a chronic disease that affects more than 135 million people worldwide[1]. Although this disease is no longer fatal, thanks to the discovery and commercial production of insulin, it does place a heavy burden on the afflicted individual, as well as their family and the healthcare system. A recent report published by the American Diabetes Association stated the total estimated cost of diabetes in America alone to be $174 billion[2] as of 2007. This amount is up $44 billion from the previous report in 2002[3]. This staggering healthcare bill breaks down to every individual diagnosed with diabetes paying $11,744[2] in additional healthcare costs due to their diagnosis. This economic burden is due to the current population of diagnosed diabetic patients reaching 17.5 million, an increase of 5.4 million from 2002. With predictions that diagnoses of diabetes will increase to 29 million in America alone by 2050[4], the cost of diabetes treatment will almost double. Diabetes Mellitus is becoming a worldwide epidemic that will affect over 300 million diagnosed individuals worldwide[1] by the year 2025.

Not only does diabetes cost society in regards to medical expenditures, diabetes also drains the economy of productivity[2]. In 2007, the total loss of productivity due to diabetes totaled $58 billion, with major contributors such as reduced productivity while at work for the employed and lost productive capacity due to early mortality totaling $46.9 billion[2]. As the incidence of diabetes increases in the future, the added burden on society as well as the individual will continue to become more and more evident.

For those individuals whose diabetes is uncontrollable by conventional treatment methodologies, such as intensive insulin administration, everyday life can become very challenging. A major complication of Type 1 Diabetes Mellitus is hypoglycemic unawareness[5], in which the diabetic patient loses consciousness due to low blood sugar. This almost always requires external help to regain consciousness, and can happen unexpectedly. This looming threat interrupts the day to day life of a diabetic individual, often affecting their work. For these individuals, treatment options that control hypoglycemic unawareness are limited.

Diabetes and Transplantation

History

Diabetes has been present as a disease since the beginning of recorded human history. Early civilizations, such as the Egyptians, Babylonians, and the Assyrians recognized the symptoms of the disease, but had no method of treating the disease. Even the father of medicine, Hippocrates, was familiar with the condition, but did not study it as it was a rare and incurable disease. In the era of the ancient Greeks, it was known for the excess amounts of urine produced by the affected individual. During the time of Hippocrates and Aristotle, diabetes was generally known as “wasting of the body”, and was not of much interest[6]. Aretaues of Cappadocia was the first person to study the disease, and is credited with the naming of the disease for the copious amounts of urine produced. The physician Avicenna later described diabetes further in his writings. He described primary symptoms as well as secondary symptoms just as Aretaues, but Avicenna was able to treat diabetes with a mixture of herbs and seeds to prevent the excretion of sugar in the urine[7]. He also described diabetic gangrene and diabetes insipidus for the first time. In 1425, diabetes was described in English

from the Greek word meaning “to pass through or siphon”. Early history of diabetes has many references to the sweetness of the urine produced by the afflicted individual. Some cultures believed that diabetes was a disease of the rich, due mainly to gluttony[6]. In 1672, Mellitus was added to the name of the disease to describe the sweetness of urine. Mellitus is the Latin word for honey. However, there was no proof that diabetic individuals were secreting sugar in their urine. Beginning in the late 1700’s, many new discoveries about diabetes were occurring. In 1776, the isolation of sugar from diabetic urine was first discovered[8]. Johann Peter Frank was the first physician to clinically differentiate Diabetes Mellitus from Diabetes Insipidus, although both forms of the disease had been described beginning with Aretaues.

As the 1800’s began to end, two physicians, Von Mering and Minkowski, discovered that removal of the pancreas in a dog led to diabetes and eventual death[9]. For the next thirty years, the pancreas remained as the cause of disease. Some physicians experimented with removal and/or replacement of the pancreas in remote locations throughout the body[10] in an attempt to cure diabetes. This first experiment in xenotransplantation was a cataclysmic failure

due to mechanisms beyond their understanding. To date, there is still much research ongoing into the field of xenotransplantation. However, this clinical experiment is important to the latter birth of transplantation.

Not until 1922, when Banting and Best[11-14] discovered that the lack of insulin was the cause of diabetes, was there any feasible treatment for diabetes. They were the first to isolate insulin and were instrumental in the widespread production of insulin. For the first time, diabetes was no longer an acutely fatal disease. Insulin was now available, as a preparation of bovine origin, as a treatment for diabetes. Early insulin injections had varying effects due to the variable purity of the preparation[15]. Over the ensuing, years many advances in the exogenous production of diabetes were made, such as long lasting insulin. In the 1950's, the first oral anti-diabetic drugs became available for patients with Type Two Diabetes Mellitus. Unfortunately, the control of blood glucose concentrations is not always effectively controlled by exogenous insulin injections; and therefore requires frequent and diligent monitoring of blood glucose and careful dosing of insulin by the individual. As a result, many diabetic individuals suffer from frequent episodes of hypoglycemic unawareness.

Prior to 1966, when the first whole organ pancreas transplant was performed at the University of Minnesota[16], the only form of treatment for diabetes was the injection of exogenous insulin. The success of simultaneous kidney[16] pancreas transplantation has provided a curative treatment for severely diabetic patients. Eventually, pancreas alone[17] transplants became more successful and abundant, further expanding the treatment options for Diabetes Mellitus. The idea of transplanting Islets of Langerhans into a diabetic recipient originated with Paul Lacy in 1972[18]. This concept arose because of the surgical complications associated with pancreas transplantation, which often proved to be fatal. However, islet transplantation did not experience much success due to the difficulty in isolating the islet cells, and the use of diabetogenic immunosuppressive drugs such as steroids. Fortunately, researchers did not give up on islet cell transplantation and pursued the improvement of the isolation process and success rate.

The initial difficulty in islet cell isolation experienced by researchers was due to the cellular architecture of the pancreas. The pancreas is an organ that has two primary functions. First, the pancreas produces exocrine enzymes that aid in digestion. These

enzymes are secreted from acinar tissue into the main pancreatic duct, which drains into the duodenum. The second function of the pancreas is the endocrine activity. The pancreas has cellular clusters, known as islets of Langerhans which are responsible for secreting hormones directly into the vasculature, which are essential to metabolism.

The islets of Langerhans are highly vascularized and made up of five cellular components (Figure 1.1). The alpha (α) cells are responsible for producing glucagon,

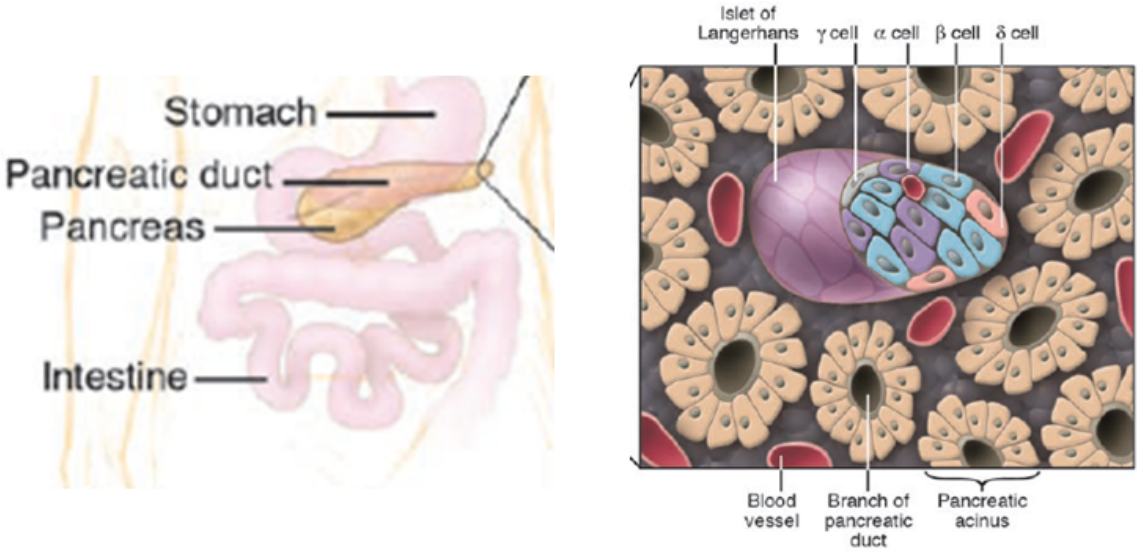

Figure 1.1 Structure of the Human Pancreas. The human pancreas consists of both exocrine (acinar) tissue and endocrine (islet) tissue. Islet cells have a microvasculature that supplies the islet cells with blood for glucose monitoring and oxygen supply. The endocrine hormones are also secreted directly into the blood supply by the capillaries present in the islets. Islet cells consist of α, β, δ, γ, and ε cells (not shown) which produce glucagon, insulin, somatostatin, pancreatic polypeptide, and ghrelin respectively. (Republished with permission from Trucco M[19].) Copyright (2005), American Society for Clinical Investigation. Permission conveyed through Copyright Clearance Center, Inc.

which is responsible for increasing blood glucose concentrations by stimulating glycogen release in the liver. The beta (β) cells are responsible for producing insulin, which facilitates the intake of glucose into cells. The third cell type is the delta (δ) cell which produces somatostatin, a hormone that regulates the endocrine system. The fourth cell type is the PP cells (sometimes known as γ cells), which produce pancreatic polypeptide, which regulates both exocrine and endocrine secretion activities within the pancreas. A fifth cell type is the epsilon (ε) cell which produces ghrelin, a hormone involved in the stimulation of the appetite. Pancreatic islets cells differ in morphology and content between species. In rodents, islet cells are highly organized with a β cell core and an α cell ring surrounding the β cells. In humans, this level of organization appears to be lost (Figure 1.2).

The human pancreas contains about one million islet cells, each of which is composed of about one thousand cells of different types[20]. Islet cells are not a uniform cell type, as they vary in size and composition. The size variance of human islet cells adds difficulty to evaluating the quantity of islet cells isolated from a human pancreas. Typically, when counting islet cells, one sees islets that

vary in size between fifty micrometers and over four hundred micrometers. For this reason, a method for

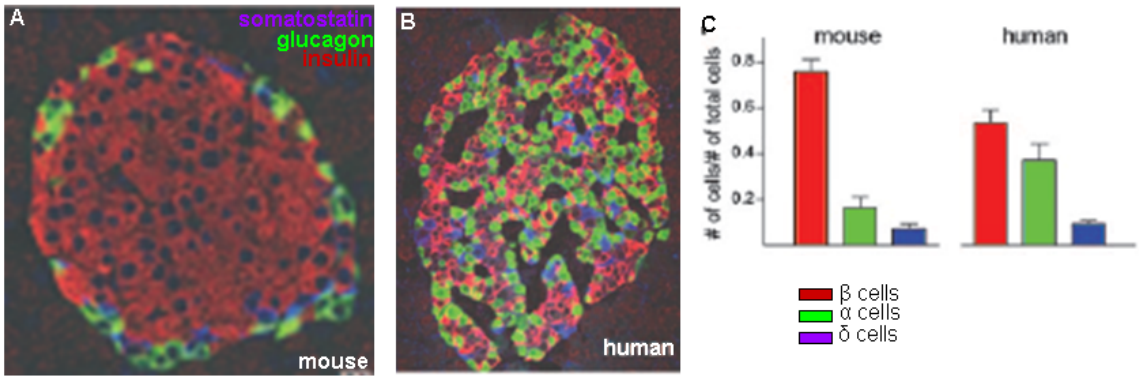

Figure 1.2 Architecture of Islet Cells Differ Between Species. Mouse islets have a distinct architectural structure within the islet (A). Beta cells are surrounded by alpha and delta cells. Human islet cells appear to have lost the organization present in rodent islet cells (B). The proportion of α, β, and δ cells also differs between species (C). Human islets have a higher percentage of α cells compared with mouse islet cells. (Republished with permission from Cabrera et al[20].) Copyright (2006), National Academy of Sciences, USA.

determining the amount of average sized (150μm) islet cells was established, called the Islet Equivalent (IEQ). Table 1.1 describes the endocrine contents of the human pancreas by size.

Table 1.1 The Islet Content of Human Pancreas[a]

Islet Diameter (μm)	Number of islets	% of total number of islets	% of total islet volume
<50	782,000	52.0%	5.4
50-100	416,000	28.0%	17
100-150	203,000	14.0%	30
150-200	54,000	3.6%	26
200-250	20,000	1.3%	15
>250	3,000	0.2%	6.3

[a]Adapted from Korsgren et al[21]. Copyright 2006, Society of Transplant Surgeons.

Etiology

To date, there is no known cure for type 1 diabetes mellitus (T1DM) and the etiology is still not clear; however, it is believed to be caused by two compounding factors. The first, and probably the most important, is genetic susceptibilities found within the human leukocyte antigen (HLA) alleles. The second is unknown environmental factors, that coupled with a genetic predisposition, culminate in autoimmune diabetes. Moreover, it is widely known that T1DM is an autoimmune disorder involving both CD4+ and CD8+ autoreactive T lymphocytes that target the destruction of the pancreatic beta cells of the islet[22]. How these T lymphocytes become autoreactive is the subject of much research. Currently, there are six known autoantigens that are targeted by the autoreactive T cells. They are islet cell autoantibody (ICA)[23], insulin autoantibodies (IAA)[24], autoantibodies against the 65 kD isoform of glutamic acid decarboxylase (GAD65)[25], the protein tyrosine phosphatase-related IA-2 molecule (IA-2A)[26,27], an antibody against a sialic acid containing glycolipid[28], and most recently an antibody against the zinc transporter ZnT8[29]. Of these six known autoantigens, it is not known if one or any of these play a more prominent role in the induction of type 1 diabetes[22]. Moreover, recent

reports suggest that more autoantigens are possibly targeted during diabetes[30].

Histocompatibility has been associated with risk for diabetes for quite awhile, with certain HLA haplotypes[31] being genetically predisposed to disease. The most important genes predisposing the individual to diabetes are the HLA DQ8 and DQ2 alleles, located on the short arm of chromosome 6, with greater than 90% of type 1 diabetic patients being positive for either or both genes[32,33]. Recent reports are now beginning to link certain HLA haplotypes with individual autoantigens. The insulin autoantigen is suggested to be strongly associated with the haplotype of HLA DR4—DQ8 in diabetes susceptibility[34], and is known to be the earliest autoantibody formed[33] during the preclinical phase of diabetes. The recognition of the insulin A-chain by the HLA DR4—DQ8 T lymphocyte requires post-translational modification of human insulin[35], suggesting that unmodified insulin is not a source of autoantigen. It has also been reported that GAD65 autoantigen is associated with the haplotype HLA DR3—DQ2[31], which is another early autoantibody formed in the preclinical phase[33].

As research into the etiology of T1DM continues, the answer seems to become more evasive. We now know that T1DM

involves autoreactive CD4+ and CD8+ T cells that are primed against autoantigens that destroy beta cells by IL-2, IFN_{γ}[36], and perforin[37] respectively. Why and how the T cells are able to avoid the mechanisms for preventing autoimmunity remain to be discovered. In the meantime, ongoing research into improved methodologies for the treatment of T1DM continues to expand.

Pancreas Transplantation

In 1967, the first vascularized pancreas transplant was performed[38]. Since the first pancreas transplant over forty years ago, much progress has been made in surgical technique, and immune suppression prolonging graft and patient survival[39]. As of 2004, there have been 23,000 pancreas transplants worldwide[40]. Historically, there are three types of pancreas transplant: Simultaneous pancreas kidney (SPK), pancreas after kidney (PAK), and pancreas transplant alone (PTA). These three alternative transplantation methods have not shared the same success rates, with SPK having a 1 year survival rate of 85%, PAK 78%, and PTA 77% as of 2003[40]. The main obstacle that had to be overcome in the early days of pancreas transplantation was the location of the pancreatic exocrine drainage, and the solutions were enteric or bladder

drainage. Enteric drainage (intestinal) is the most physiologic of the two methods[40]. This method is superior to bladder drainage in respect to metabolic dysfunction, urinary tract infections, and pancreatitis, but has a higher risk for infections due to enteric leaks. The bladder drainage technique has less severe risk complications, and can be easily monitored by urinary amylase content. Historically, the bladder drainage technique was more prevalent during the 1990's. However, enteric drainage has become the predominant technique used[41] in recent years.

Early Islet Transplantation

Islet cell transplantation has been in development as a means of treatment for type 1 diabetes for several decades. The field of islet transplantation began in 1972 when Lacy et al proposed isolating and transplanting the source of insulin producing cells - the islets of Langerhans[18]. Many researchers at the University of Minnesota began experimenting with the technique for isolating islets for the purpose of transplanting the islets in various locations within patients[42].

Many improvements to the isolation technique were made during the subsequent decades, which improved the islet

yield and purity. Some of these improvements were: 1) the use of density gradients to separate the endocrine tissue from the exocrine tissue[43], 2) the injection of collagenase into the pancreatic duct[44], and 3) the development of the semi-automated method[45]. The development of the semi-automated method was of great importance because it standardized the method of islet cell isolation. Since the introduction of the semi-automated method, all islet cell isolation centers follow some derivative of the original method developed by Ricordi. Although the semi-automated method standardized the isolation of pancreatic islet cells, making the recovery of islet cells reproducible and efficient, it did not improve the clinical outcome in patients. Because the achievement of insulin independence, the primary endpoint of the studies, was less than 10% at one year post-transplant, many transplant centers were reluctant to use this method over whole pancreas transplantation as a treatment of autoimmune diabetes.

A New Beginning

The Edmonton Protocol

The publication of the results from the islet transplant center in Edmonton, Canada in 2000 changed the field of islet cell transplantation. Their initial report

had a 100% success rate of reversal of diabetes as measured by insulin independence one year after transplant[46]. This is mainly attributed to their avoidance of steroids, a staple immunosuppressive agent in whole organ transplantation, which are known to be diabetogenic in nature[47]. In addition to steroid removal, the Edmonton group used a protocol that included sirolimus, low dose tacrolimus, and daclizumab (a monoclonal antibody against the interleukin-2 receptor) in islet cell transplantation alone for the treatment of brittle type 1 diabetes. The Edmonton protocol also included transplantation of a minimum of 10,000 IEQ/kg in order to achieve insulin independence. This required multiple infusions from more than one donor to attain adequate islet mass to maintain insulin independence.

Edmonton's success in achieving 100% insulin independence, when previous groups had <10% success[48,49], stimulated a renewed interest in the subject. Many new islet transplant centers, including the Baylor Islet Cell Transplant program, began to emerge over the next few years as a result. Established islet transplant centers were able to duplicate the results of the Edmonton group with their own patients experiencing a similar success rate[50]. An international trial of the Edmonton protocol, involving

eleven established and new centers, was underway to test the reproducibility of Edmonton's results. This trial combined centers with many years of experience with centers that were relatively inexperienced. The combined result was an insulin independence success rate of 28% at one year, and a total insulin independence of 58%[51]. Researchers at the University of Minnesota began investigating the possibility of performing single donor islet transplantation for the treatment of diabetes. By using carefully selected donors and recipients, along with more potent induction immunotherapy and less diabetogenic maintenance therapy, they were able to attain an insulin independence rate of 63%[52]. Researchers at the University of Minnesota were able to demonstrate that single donor islet cell transplantation is capable of producing a sufficient engrafted β cell mass to efficiently stabilize blood glucose without exogenous insulin.

Baylor Islet Cell Program

Our islet transplant program began in 2003 originally as a remote site isolation transplant center. Initially, we collaborated with the University of Miami School of Medicine and Diabetes Research Institute for the isolation of human pancreatic islet cells. During this time of

remote site transplantation, our team worked on validating our own isolation process with the FDA, finally getting approval in 2006. Since the FDA approval of our laboratory we have performed 80 research islet isolations and nine clinical transplants. Seven out of nine patients achieved insulin independent status. However, five of these patients had transient insulin independent status and have returned to insulin use, although at a significantly reduced dosage. All of the islet transplant patients have shown no episodes of severe hypoglycemic unawareness. Initially, our program followed the Edmonton protocol for immunosuppression and patient follow up. Since then, our program has had its own innovative protocol approved.

Challenges Facing Islet Transplant Centers

Shortage of Donors

Donor selection for the isolation of islets of Langerhans for clinical transplant is very stringent. The requirements of optimal donor organs for both whole organ transplant and islet cell transplant make islet cell transplantation a second priority to whole organ transplantation. However, this usually translates into sub optimal organs that were rejected for whole organ transplant being used for islet cell transplantation.

There have been groups that have ventured into the realm of marginal donors and experienced success[53,54]. This opens the possibility of expanding the donor pool for islet cell transplantation, with the effect of increasing the number of islet cell transplants.

Efficiency of Isolation

Currently, it is believed that there are an average of 1 million islet equivalents in a human pancreas. The current isolation procedures are, at best, able to recover consistently 50% of the total islet mass. Coupled with the observation that only 25 to 50% of the transplanted islet mass is engrafted[55], multiple donor pancreata are required to produce an adequate islet mass that will sustain insulin independence and glycemic control in just one recipient. Improvements need to be made in islet purification and collection so that greater than 50% total islet yield can be recovered from the donor pancreas, so that single donor islet transplantation becomes the mainstay rather than an anomaly.

Islet Quality Prior to Transplant

Many centers are experimenting with culturing islets in the presence of different growth factors[56], antioxidants[57], endothelial cells[58], and recombinant viral

vectors[59-61] all with the same goal in mind - to increase the survival of the transplanted islet mass so that one donor organ could be used to treat one patient or even multiple patients. This would greatly increase the availability of the procedure to a larger population of diabetic patients.

Improving Islet Engraftment

Due to the small islet mass that survives the transplantation procedure into the hepatic portal vein, multiple islet transplants are needed at most centers to reach the primary end point of insulin independence. Preventing the destruction of the transplanted islets has been an area of abundant research recently. One of the major obstacles to the success of islet transplantation is the instant blood mediated inflammatory reaction (IBMIR)[62], which is thought to be responsible for the loss of the majority of islets post-transplant. The exact mechanisms of IBMIR are not fully understood, but it is believed that the interaction of ABO compatible blood with allogeneic islets causes the release of pro-inflammatory cytokines from either the islets themselves or components in the blood. It is also known that islets express membrane bound tissue factor, as well as the alternatively spliced soluble form. Tissue factor has been demonstrated to elicit a

strong thrombotic response when islets come in contact with ABO compatible whole blood[63]. Preventing the destruction of islets during the post-transplant period is the subject of much research. To date the mechanisms of islet destruction are not fully understood. There is some emerging data suggesting that islets are undergoing apoptosis caused by many factors[64], as well as IBMIR.

Overcoming the Immune Response

Furthermore, currently used methods of immunosupression are not without consequences. There has been documentation of many side effects[65], and it is well known that high doses of rapamycin can have a negative effect on islet cell engraftment[66], inhibit insulin signaling[67], cause insulin resistance[68], and inhibit revascularization[69] and proliferation of pancreatic beta cells[70,71]. In addition, calcineurin inhibitors are known to be toxic to beta cells[72,73]. Developing a technique to induce tolerance in the recipient would have profound effects on the field of cellular transplantation and possibly solid organ transplantation. Just like any other transplant procedure, there exists the problem of immune suppression. Immune suppression was a major problem in the early stages of the development of islet cell

transplantation. Initial immunosuppressive regimens were similar to drugs used for other transplant procedures, which included steroids. The advent of a steroid free immunosuppressive regimen greatly enhanced the survival of the islet mass, but this treatment is not without side effects. New methods of immune suppression are being tested that induce tolerance after transplantation[74,75], allowing for the withdrawal of immunosuppressive agents.

Current Status of Islet Transplantation

Multiple Donors Still Required to Attain Insulin Independence

Islet cell infusions from more than one donor are still a requirement to achieve insulin independence in islet cell transplant patients. There are several reasons for this. First, the isolation process often does not acquire an adequate quantity of islet cells from one donor pancreas to achieve euglycemia without exogenous insulin. The imperfect islet isolation process has many factors contributing to this including, but not limited to: collagenase enzyme batch, cold ischemia time, warm ischemia time, donor age, and donor BMI etc. Second, during the transplant procedure, the majority (up to 70%) of the transplanted islet cells are destroyed following their

contact with blood cells. Third, the islet toxic nature of the immunosuppressive drugs and the resulting impact on the islet graft. The reasons for the loss of function of the transplanted islet mass are not clearly understood, but could be attributed to beta cell exhaustion and/or immunological rejection.

Donor Specific Sensitization

Recent reports have begun to demonstrate the sensitization of past islet cell transplant recipients to previous donor specific antigens[76-81]. These reports have demonstrated sensitization to both HLA class I, present on all cell types, and HLA class II, which has restricted cellular expression. These reports also show that sensitization can occur if the patient ceases taking their immunosuppressive regimen, and more importantly do occur while the patient is still immunosuppressed. In whole organ transplantation, the development of donor specific antibodies (DSA) is generally associated with rejection of the allograft.

Focus of Dissertation

The focus of this study will be in three areas. The first topic will focus on the influence of cold ischemic organ preservation on the quantity and quality of islet

cell isolation. The second topic will focus on understanding the genetic changes islet cells initiated in response to contact with diabetic autoantibodies and complement deposition as measured by microarray analysis and real-time PCR. The third topic will focus on the up-regulation of HLA class II molecules on the islet cell surface in response to inflammatory cytokines.

Cold Ischemic Time and Islet Cell Isolation

Cold ischemic time (CIT) is generally defined as the time between organ placement in cold preservation solution and the removal of the organ for processing or enzyme digestion. Pancreas preservation plays a crucial role in the outcome of islet cell isolation and transplantation. It is the policy of many transplant centers not to accept organs for clinical islet cell isolation that have CIT of greater than eight hours if preserved in University of Wisconsin solution (UW) alone, or greater than twelve hours if preserved by the two-layer method (TLM)[82]. However, our center routinely procures organs locally, resulting in CIT of less than six hours. In our experience, our center has a higher success rate in the isolation of pancreatic islet cells meeting the release criteria for transplantation (unpublished data). These results lead us to believe that

CIT has a profound impact on the outcome of pancreatic islet cell isolation. We hypothesized that CIT of less than four hours would have a beneficial effect on the outcome of islet cell isolation, when compared to the standard eight and twelve hour limits.

Islet Cell Response to T1DM Auto-Antibodies and Complement

A major obstacle to the success of islet cell transplantation as a standard treatment for labile type 1 diabetes mellitus is the immediate loss of up to 70%[62] of the transplanted islet mass, leading to the necessity of multiple transplants. Most of the research to date has focused on the initiation of IBMIR. Reports have found that the activation of the complement cascade and coagulation factors initiates the destruction of the islet graft[62].

However, no insight into the response from pancreatic islet cells to autoantibodies and complement present in the recipient has been established. In this study, we set out to determine if autoantibodies are capable of initializing complement fixation on the surface of isolated pancreatic islet cells, and then analyze the gene expression changes in islet cells following complement activation as a result of exposure to type 1 diabetic serum (T1DM). We

hypothesized that autoantibodies present in diabetic serum are indeed capable of initiating complement fixation of the surface of the islet cell, which in turn leads to cytotoxicity and a change in the expression of islet cell genes.

HLA Expression on Isolated Pancreatic Islet Cells

Recent reports have shown that islet transplant recipients develop antibodies against donor HLA class I and II[77,79,80]. In addition to the published reports, our center has had two out of nine patients develop antibodies against donor HLA, in particular HLA class II. HLA class I is ubiquitously expressed on all nucleated cells, while HLA class II is generally restricted to antigen presenting cells (APC). Since human islets do not express HLA class II under normal conditions, and mechanisms underlying induction of the anti-class II response are unclear; we were curious as to how our patients developed antibodies against HLA class II.

Previous reports have shown that certain cell types can be induced to express HLA class II on their cell membrane under inflammatory conditions[83-85]. Therefore, we hypothesized that under inflammatory conditions, islet cells will have induced expression of HLA class II.

CHAPTER TWO

Prolonged Cold Ischemia has a Detrimental Effect on the Outcome of Islet Cell Isolation

Introduction

Since the success of the steroid free protocol introduced by the Edmonton group in the year 2000[46], islet cell transplantation has become a more practiced method of treatment for type 1 diabetes mellitus. Edmonton's success encouraged several established transplant centers, including ours, to start their own islet cell transplant centers to treat local diabetic patients. The results of the Edmonton protocol were well reproduced throughout centers all over the world[51]. However, islet processing centers are still sparsely distributed, resulting in many transplant quality organs originating from distant procurement agencies.

The isolation of human pancreatic islet cells requires highly skilled technicians and is a long process. The isolation begins with the organ procurement surgeon performing the pancreas recovery. Because islet transplantation often uses organs that are not fit for solid organ transplantation, the procurement surgeon often

has to wait until the other organs for transplant are removed. The pancreas is packed with ice to prevent warm ischemia, while the vasculature is clamped off. Removal is technically difficult and requires the skill of a surgeon for proper removal. Often, the decision for islet transplantation is made after the organ is removed for whole organ transplantation and rejected for various reasons. The pancreas alone or in combination with the spleen and duodenum is then placed into organ preservation solution and kept on ice until arrival at the isolation center (Figure 2.1A). Once the pancreas arrives at the

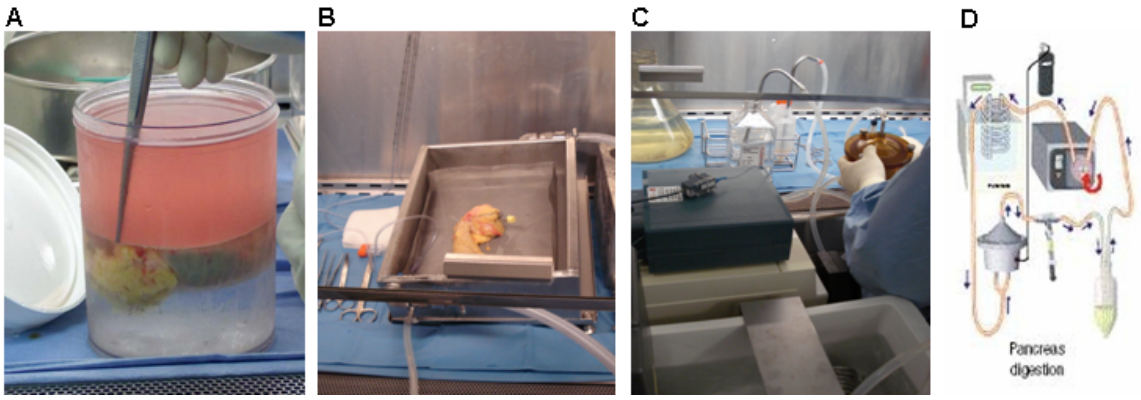

Figure 2.1 Pancreas Cleaning, Perfusion, and Digestion. Cadaveric donor pancreata are transported to the Baylor islet cell processing laboratory for the isolation of pancreatic islet cells. The donor pancreas is cleaned of excess fat and any extra organs are removed (A). After the organ is decontaminated, the cannulated organ is placed on a perfusion apparatus where the rate of enzyme solution infusion and the pressure of the organ are kept constant (B). Once the perfusion of the enzyme is complete, the pancreas is cut into small pieces and placed in a Ricordi chamber (C). The Ricordi chamber is then connected to a circuit to circulate and change the temperature of the enzyme during the digestion phase of isolation (D).

isolation center, it needs to be cleaned of excess fat and have the spleen and duodenum removed. The cleaning of the pancreas requires proper training, but can be performed by non-surgically trained technicians. Upon completion of pancreas cleaning, the pancreatic duct is cannulated, and the pancreas is dipped into betadine and antibiotic solutions for decontamination. The pancreas is digested by perfusing a chilled collagenase enzyme through the pancreatic duct, while maintaining a constant pressure (Figure 2.1B). The perfusion of the pancreas causes the distension of the pancreas, enabling further removal of surface fat. The enzyme is allowed to perfuse for ten minutes before the pancreas is placed into the digestion chamber. The pancreas is cut into small pieces and placed in the digestion chamber along with seven hollow stainless steel marbles to aid in mechanical digestion (Figure 2.1C).

The digestion chamber is shaken gently, and the fluid part is circulated within a circuit designed to warm the enzyme (Figure 2.1D). Samples are taken at short intervals to monitor the digestion process. When the digestion is deemed to be finished, the enzyme is cooled and diluted by adding chilled dilution solution into the circuit. The resulting digested tissue is collected into many conical tubes containing human serum albumin (HSA). The tubes are

spun down and the pellets are pooled together into one tube containing UW solution. The soaking of the tissue in UW solution causes disparate dehydration of the acinar tissue and the endocrine tissue. The digested pancreatic tissue is enumerated for islet cells by taking a small (100μl) aliquot from a known volume and stained with dithizone, a dye specific for zinc present in beta cells (Figure 2.2A). The remaining tissue is then purified using solutions forming a continuous gradient and centrifugation using a COBE cell processor. The purified tissue is collected from

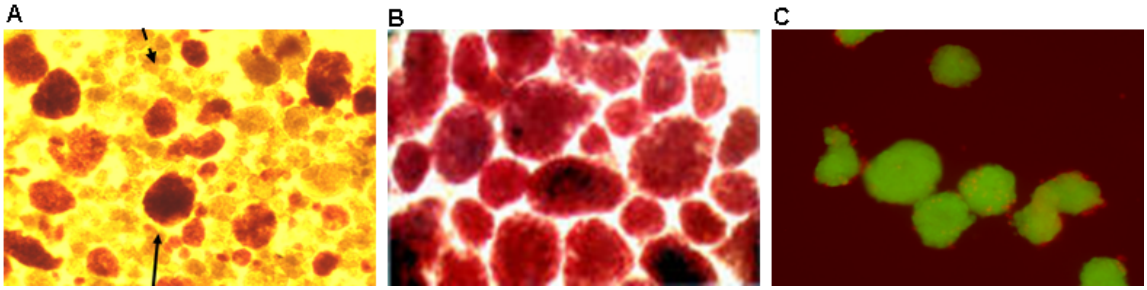

Figure 2.2 Islet Cell Counting and Viability Assessment. After the pancreas is digested the cellular content is collected and a count of the endocrine cell is performed using a zinc specific dye (dithizone) that stains beta cells red (A). Solid arrow indicates islet cell (endocrine), broken arrow indicates acinar (exocrine) tissue. The endocrine tissue is then purified based upon density gradient centrifugation in a COBE cell processor. The purified cellular contents are then counted again using dithizone (B) and purity is assessed. Upon completion of the purification process, a small aliquot of the purified preparation is taken for the assessment of viability using fluorescein diacetate (FDA, green) and propidium iodide (PI, red) (C).

the COBE cell processor in 25ml aliquots and placed into ten conical tubes filled with dilution solution supplemented with heparin and HSA. Small aliquots are

taken from each conical tube and evaluated for islet cell content and purity. The conical tubes are centrifuged and similar purity fractions are pooled into three purity layers. Each purity layer is then enumerated and endocrine purity is assessed using dithizone (Figure 2.2B). The purified islets are then either prepared for transplant or placed into culture. Small aliquots from each layer are taken for the evaluation of viability, using FDA/PI staining (Figure 2.2C), and potency, using glucose stimulation.

Our center initially began as a remote site transplantation center in collaboration with the Diabetes Research Institute (DRI) at Miami University School of Medicine. During this initial period, pancreata procured locally were sent to DRI for the isolation of islet cells for the purpose of transplantation. Upon completion of isolation, if the islet cell preparation met the criteria for transplantation, DRI would ship the islet cell preparation to Baylor. Of the thirteen organs sent to DRI for the purpose of transplantation, only five met the release criteria for transplantation and were transplanted into four recipients. Due to the distance between our center and DRI, cold ischemia times (CIT) were prolonged.

In 2006, Baylor was approved by the FDA as an islet isolation and transplant center. Since then, we have performed 44 research islet isolations and twelve clinical isolations resulting in nine transplants. I have actively participated in most of these isolations and performed several steps during isolation and subsequent islet quality testing. For on-site isolation, the duration of CIT varied widely depending on the location of the procurement center. More recently, pancreas procurement for islet isolations is performed by islet team surgeons who have significantly impacted islet isolation outcome. Our team has introduced a method to preserve the main pancreatic duct during cold storage, which we believe improves the outcome of isolation. Since the implementation of the novel ductal preservation technique, we have had seven consecutive transplants from eight clinical grade pancreata. For recent islet isolations, the vast majority of pancreata were processed with CIT of less than four hours.

The goal of the present study was to determine what effects prolonged CIT has on the outcome of islet cell isolation. All of the isolation data, dating back to the initiation of the remote site isolation to the present data were compiled into a spreadsheet (Table 2.1). Our hypothesis was that prolonged cold ischemia time (CIT)

adversely affects the outcome of islet cell isolation. To this end, we evaluated the isolation data based upon four

Table 2.1 Donor Characteristics

Group	CIT (hrs)	n	Sex	Age	BMI	Mean CIT
I	0 to 3:59	17	11M:6F	40.35 ± 3.16	31.04 ± 1.63	2:40 ± 0:10
II	4:00 to 7:59	18	14M:4F	42.5 ± 2.82	29.74 ± 1.40	5:29 ± 0:19
III	8:00 to 11:59	12	9M:3F	46.9 ± 3.70	31.3 ± 2.50	9:08 ± 0:17
IV	>12hrs	5	2M:3F	47.6 ± 4.40	31.2 ± 2.90	16:08 ± 1:44

predetermined CIT groupings, looking at all of the recorded islet cell evaluations estimating quality as well as quantity.

Materials and Methods

Procurement of Pancreata

Pancreas procurement for the isolation of pancreatic islet cells has occurred under three separate protocols. During protocol one, pancreata were procured by Baylor Regional Transplant Institute (BRTI) surgeons and outside surgeons. Pancreata in the north Texas vicinity were procured by BRTI surgeons following a standardized technique to minimize warm ischemia time. The pancreas was excised along with the spleen and duodenum, and placed in either University of Wisconsin (UW) solution (ViaSpan; Duramed, Pomona, NY), or in a combination of oxygenated perfluorocarbon (PFC) (Appollo Scientific, UK) and UW known

as the two later method. Preserved pancreata were then couriered to the Diabetic Research Institute (DRI) at the University of Miami School of Medicine for isolation of clinical grade islet cells, while those pancreata designated for research only were couriered to the Baylor Islet Cell Transplant (BICT) for the isolation of research grade islet cells.

During protocol two, donor pancreata were procured by BRTI surgeons and outside surgeons following a standardized technique to minimize warm ischemia followed by the UW alone or TLM methods of preservation. Preserved pancreata were then couriered to BICT for the isolation of both clinical and research grade pancreatic islet cells.

Protocol three was implemented when BICT team included its own surgeons specializing in islet cell isolation and transplantation. All pancreata were obtained by the BICT surgeons using a standardized technique to minimize warm ischemia time. BICT surgeons also implemented a novel technique to preserve the main pancreatic duct integrity, by injecting organ preservation solution immediately upon excision. Pancreata were then cleaned on the back table and placed in either UW/TLM, M-Kyoto/TLM, or Kyoto/TLM preservation solutions for islet cell isolation.

Islet Cell Isolation

Upon arrival to the BICT processing laboratory the pancreas was cleaned of excess fat, cannulated if necessary, and decontaminated in betadine followed by an antibiotic soak. Pancreata were then perfused with either Liberase (Roche, Indianapolis, IN), or collagenase NB1 (Serva, Heidelberg, Germany) enzymes for the digestion of the pancreas. Upon completion of enzyme perfusion, the fully distended pancreas was cleaned further of any remaining fat, and then cut into several pieces prior to placement in the Ricordi chamber. Pancreata digestion was carried out following the semi-automated method described by Ricordi et al[45]. Upon cessation of digestion, the digestate was diluted to reduce the enzyme activity, and collected into multiple conical tubes containing human serum albumin (HSA) (Baxter, Deerfield, IL) and dilution solution (Mediatech, Manassas, VA). The conical tubes containing digestate were centrifuged and the supernatant removed. The cell pellets were re-suspended in HSA/UW and transferred to two conical tubes. The digestate was then evaluated for islet content, digestion rate, and purity. Digestate was then purified on a COBE 2991 cell processor (Gambro BCT, Lakewood, CO) using a continuous gradient created using Biocoll (Biochrom AG, Berlin, Germany) or

Iodixanol (Optiprep; Sigma, St Louis, MO). Islet fractions were then assessed for islet cell content, purity, viability, and digestion, and pooled based upon purity.

Islet Cell Assessment

Samples from each islet cell preparation were taken for counting, purity, viability, and potency estimation. Islet cells were counted as previously described[86] using dithizone to stain the islets. Briefly, a 100μl sample of the purified islet layers is taken from 100ml of total sample and placed in a counting dish. Next, dithizone is added to the islet sample. Using an inverted light microscope, the islet cells are assessed using a micrometer to establish the size of each islet cell. The number of islets of each size group were counted and used to determine the number of islet equivalents (IEQ) present. Endocrine purity is estimated based upon the ratio of dithizone positive (red) cells compared with dithizone negative (yellow) cells. Viability was determined using fluorescein diacetate and propidium iodide (FDA/PI) inclusion/exclusion. Briefly, FDA is cell permeant and able to penetrate viable cells, staining them fluorescent green. PI is cell impermeant, and unable to penetrate viable cells, staining cells with disrupted cell membranes

fluorescent red. The ratio of green:red cells is estimated using an inverted fluorescent microscope for each islet cell. The viability for 50 IEQ is estimated, and then an average viability is determined. Islet potency was determined by static incubation. Briefly, 250 IEQ were placed in CMRL supplemented islet cell culture medium at 37°C for 18 to 24 hours after purification. 50 IEQ were then placed in duplicate into either low (2.8mM) or high (20.0mM) glucose solution, to stimulate insulin secretion. The islets were then placed at 37°C for two hours before insulin secretion was measured by ELISA. Samples were corrected for DNA content by measuring the DNA content of each sample. A stimulation index was calculated as the ratio of the amount of insulin/DNA from high glucose compared to low glucose.

Data Collection

Results from all isolations performed at BICT using all three protocols were collected and combined into one of four groups. Group I contained all isolation data with CIT of less than four hours. Group II contained all isolation data with CIT of greater than four hours but less than eight hours. Group III contained all isolation data with CIT of greater than eight hours but less than twelve hours.

Group IV contained all data with CIT of greater than twelve hours. Further matching based on digestion enzyme, gradient, and ductal preservation used was performed prior to statistical comparison.

Statistical Methods

All data is expressed as mean ± SEM. One way and two way ANOVA analysis were used to detect statistical significance within the CIT groups. Post ANOVA testing was performed after two-way ANOVA analysis using the Bonferroni calculation. Two-tailed students t-test was used to determine statistical significance between Groups I and II. Data was considered to be significant when $p<0.05$.

Results

Prolonged Cold Ischemic Time Reduces the Quantity of Isolated Islet Cells

To establish what impact cold ischemia time has on the outcome of human pancreatic islet cell isolation, I retrospectively analyzed all of the isolation data obtained through the Baylor Islet Cell Transplant Center. All of the isolation data was compiled into one of four groupings based upon cold ischemia time, as described in materials and methods. One way ANOVA analysis revealed no statistical significance between the CIT groups in regards

to the donor characteristics (Table 2.1). There was no statistical significance observed for donor age (40.35 ± 3.16, 42.5 ± 2.82, 46.9 ± 3.7, 47.6 ± 4.4) or for donor BMI (31.04 ± 1.63, 29.74 ± 1.4, 31.3 ± 2.5, 31.2 ± 2.9) among groups I, II, III, and IV respectively. However, ANOVA analysis (Table 2.2) of the isolation data demonstrated

Table 2.2 One way ANOVA analysis of Isolation Data

		Pre-Purification (IEQ)		Post-Purification (IEQ)		IEQ/g		% Recovery	
Group	n	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
I	17	751,333	76,059	558,569	56,744	5,512	641	77.87	8.24
II	18	501,186	54,887	356,093	77,008	3,690	846	60.96	8.87
III	12	499,153	65,946	285,696	48,388	2,804	536	55.23	4.43
IV	5	353,471	104,951	256,211	77,183	3,535	901	64.63	18.21
p		0.007		0.02		0.08		0.28	

significant impairment of islet liberation between the four cold ischemia groups (group I: 751,333 ± 76,059; group II: 501,186 ± 54,887; group III: 499,153 ± 65,946; group IV: 353,471 ± 104,951) for pre-purification IEQ. There was no statistical significance observed among the CIT groups for islet cell recovery or IEQ/g digested pancreas. Further statistical analysis between groups I and II revealed a significant reduction in islet liberation as the cold ischemic time increased (Figure 2.3A).

Because prolonged cold ischemia had a negative outcome on the liberation of isolated islet cells from intact pancreata, I examined the result of CIT on the purification of human pancreatic islet cell yield. One way ANOVA

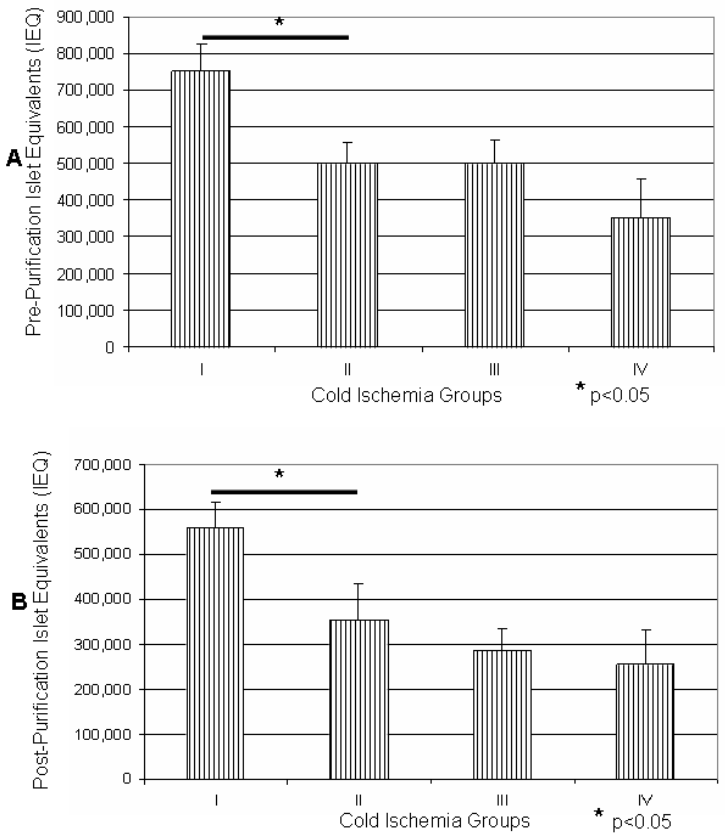

Figure 2.3 Prolonged Cold Ischemia Adversely Influences Islet Cell Liberation and Purification. All isolation data was grouped by cold ischemic time (CIT). Group I, CIT<4 hrs; group II, 4≤CIT≤8 hrs; group III, 8 ≤CIT≤12 hrs; group IV, CIT>12 hrs. One-way ANOVA analysis of pre-purification islet quantity (A) demonstrated significant improvement of islet cell liberation in CIT group I when compared to the other groups (p=0.007). Further statistical analysis of CIT groups I and II, revealed a significant improvement in CIT group I over group II (p=0.01). One-way ANOVA analysis of post-purification islet quantity (B) demonstrated a significant improvement of islet cell purification in CIT group I when compared to all other CIT groups (p=0.02). Further statistical analysis of CIT groups I and II, revealed a significant improvement of islet cell purification in group I compared with group 2 (p=0.044).

analysis demonstrated a significant inhibition of the isolation of purified pancreatic islet cells when comparing all CIT groups (group I: 558,569 ± 56,744; group II: 356,093 ± 77,008; group III: 285,696 ± 48,388; group IV:

256,211 ± 77,183) (Table 2.2). Further statistical analysis of groups I and II revealed a significant inhibition of the isolation of pancreatic islet cells as the length of cold ischemia increased (Figure 2.3B).

Taken together, these data indicate that a cold ischemic time of less than four hours significantly increases the liberation and purification of pancreatic islet cells when compared to the other cold ischemic groups. One can also conclude that CIT of less than four hours results in significantly more isolated islet cells than the traditional CIT of eight hours or less.

Prolonged Cold Ischemic Time Influences the Effectiveness of Enzyme

Two-way ANOVA analysis of pre-purification data from groups I and II compared on the basis of the brand of enzyme used demonstrated a significant increase in islet liberation between groups I and II, and a significant improvement between Serva and Liberase enzymes (Figure 2.4A). Post analysis testing showed a significant improvement in islet liberation (858,694 ± 96,656; 554,506 ± 79,276; $p<0.05$) using Serva in contrast to the use of Liberase in group I respectively, while the use of Serva or Liberase in group II had no statistical significance on pre-purification IEQ (Table 2.3).

Table 2.3 Two way ANOVA Analysis of Isolation Data

	Pre-Purification (IEQ)							Post-Purification (IEQ)						
	Liberase			Serva				Liberase			Serva			
Group	n	Mean	SEM	n	Mean	SEM	p	n	Mean	SEM	n	Mean	SEM	p
I	6	554,506	79,276	11	858,694*	96,565	0.0057	6	455,582	110,237	11	614,744	61,266	0.41
II	11	428,977	62,906	8	608,613	78,047		11	210,314	50,879	8	565,276**	130,311	
p				0.002							0.08			

*p<0.05, **p<0.01

Two-way ANOVA analysis of post-purification data from groups I and II demonstrated a trend towards significant inhibition of the isolation of purified pancreatic islet cells as the length of cold ischemic organ preservation increased, but showed no statistical significance between the brand of enzyme used (Figure 2.4B). However, post-testing analysis revealed that there was a significant increase in purified islet cells (565,276 ± 130,311; 210,314 ± 50,879; p<0.001) when using Serva compared with the use of Liberase in group II respectively (Table 2.3). Interestingly, CIT appeared to have no significant affect on the quantity of purified pancreatic islets isolated compared to the weight of the digested pancreas (IEQ/g), or on the percent recovery from the pre-purification count. Taken together these data indicate that prolongation cold ischemic organ preservation significantly reduces the quantity of purified pancreatic islet cells isolated. Moreover, the use of Liberase enzyme significantly reduces

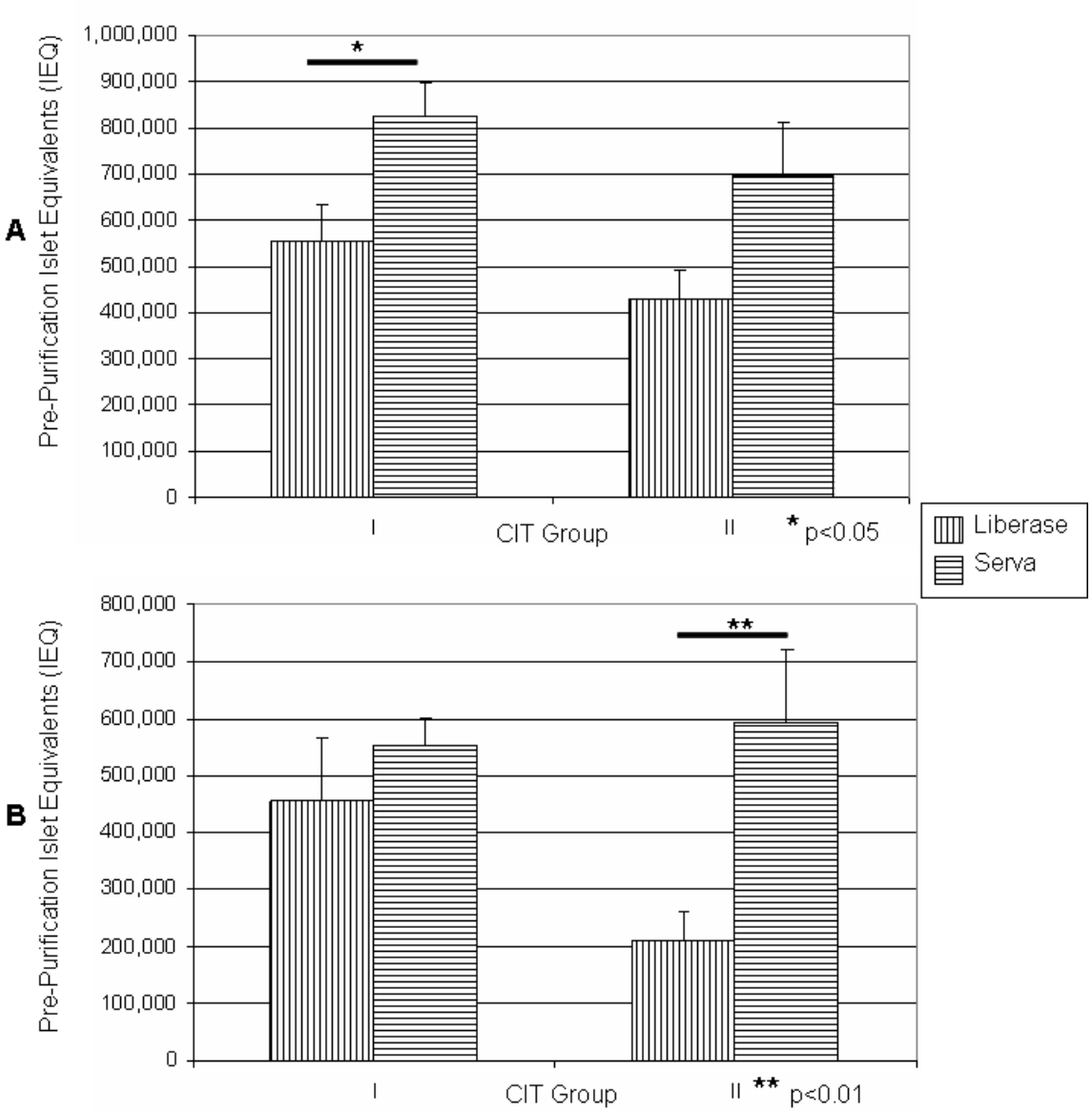

Figure 2.4 The Length of CIT and the use of Enzyme Significantly Affects the Islet Cell Liberation Yield. Islet cell data was divided into two groups based upon cold ischemic time (CIT); Group I, CIT<4 hours; Group II, 4≤CIT≤8 hours. Each group was further divided, based upon enzyme used, into two groups Liberase and Serva. Two-way ANOVA analysis of the pre-purification data (A) revealed a significant difference between CIT groups ($p=0.002$) and between enzyme groups ($p=0.0057$). Further post ANOVA testing revealed that the use of Serva in group I has a statistically significant improvement over the use of Liberase ($p<0.05$). Two-way ANOVA analysis of post-purification data (B) revealed a trend toward significant difference between CIT groups ($p=0.08$), but showed no difference between enzyme groups ($p=0.41$). Further post ANOVA testing revealed that the use of Serva in group II has a statistically significant improvement over the use of Liberase ($p<0.01$).

the quantity of purified pancreatic islet cells when cold ischemic organ preservation exceeds four hours.

Prolonged Cold Ischemia Adversely Affects the Quality of Isolated Islets

To establish the qualitative outcome of prolonged cold ischemic storage on isolated islet cells, I retrospectively analyzed the qualitative assessments of the isolation data. Again, the data was compiled into four groupings based upon cold ischemia time as described in the materials and methods section. Extended periods of cold ischemia detrimentally changed the viability of the resulting islet cells (Table 2.4). ANOVA analysis demonstrated a trend

Table 2. 4 One way ANOVA Analysis of Islet Cell Quality

		Viability		Purity		Potency	
Group	n	Mean	SEM	Mean	SEM	Mean	SEM
I	17	95.89	0.91	55.85	4.14	4.14	0.97
II	18	93.37	2.41	57.87	5.10	3.19	0.84
III	12	90.43	1.82	56.63	6.16	1.78	0.46
IV	5	87.65	1.96	62.38	8.43	1.87	0.41
p		0.09		0.94		0.25	

toward significance in the reduction of the viability (95.89 ± 0.91, 93.37 ± 2.41, 90.43 ± 1.82, 87.65 ± 1.96; p=0.09)of purified pancreatic islet cells as the length of cold ischemic organ preservation increases immediately after the isolation process for groups I, II, III and IV

respectively. However, post-purification islet purity (group I: 55.85 ± 4.14; group II: 57.87 ± 5.10; group III: 56.63 ± 6.16l; group IV: 62.38 ± 8.43) and potency (group I:4.14 ± 0.97; group II: 3.19 ± 0.84; group III: 1.78 ± 0.46; group IV: 1.87 ± 0.41) were not adversely affected by prolonged cold ischemic time groups (Table 2.4). Taken together, these data imply that long periods of cold ischemia during organ preservation show a trend toward significant increase in the quantity of dead islets at the end of the isolation process, adversely affecting the quality of the finished product available for transplantation use.

Discussion

Previous reports have shown that the use of UW alone as the preservations solution is able to maintain islet cell quantity up until eight hours, while the use of the TLM is able to prolong cold preservation up to twelve hours[82]. The advantage of the use of the TLM for preservation is debatable; many reports have shown a clear advantage[87-89], while others have shown no difference between the pancreas preservation methods[90]. Although my data was insufficient to draw conclusions regarding the benefit of TLM over UW alone in organ preservation, it clearly

demonstrates that cold preservation has a significant impact on the islet isolation process. Not only does my data demonstrate a significant improvement in the quantity of islet cells isolated and purified within cold preservation times of less than four hours compared with preservation times of four to eight hours, eight to twelve hours, and more than twelve hours, my data also demonstrates a significant difference between preservation times of less than four hours and four to eight hours alone. This is important because no previous reports have compared cold ischemia times of less than eight hours. Previous reports demonstrate a significant difference between cold preservation of less than eight hours compared with cold preservation of greater than eight hours[82].

My data also suggests that the brand of enzyme used also contributes significantly to the quantity of islet cells digested from the intact pancreas and the quantity of purified islets recovered during the isolation process. These results are in contrast to a previous report showing no significant difference between the use of Liberase or Serva Collagenase for the enzymatic digestion of the pancreas[91]. However, this report used pancreata with cold ischemic times of 12.0 ± 5.3 hours and 15.0 ± 5.7 hours, respectively. It is plausible that with prolonged cold

ischemic injury, the beneficial effect of Serva Collagenase is lost on isolation outcome. Unfortunately, all of our data pertaining to cold ischemic time greater than eight hours only used one enzyme, Liberase. It would be of interest to see if our results would match or refute those results reported by Sabek et al[91].

These data also indicate that for optimal islet cell purification, at least in groups I and II, Serva collagenase should be used in place of Liberase. This data is useful in planning the set up of pancreatic islet cell isolation, in that if the CIT can be estimated ahead of time the better enzyme would be used. Unfortunately, this study was unable to determine any statistical difference in enzyme effectiveness when the CIT was greater than eight hours. However, as demonstrated previously[91], when the organ preservation is greater than twelve hours, there seems to be no difference in enzyme effectiveness. A secondary benefit of being able to determine the enzyme ahead of time is cost effectiveness; as Liberase is about half the price of Serva.

These data are of importance because of the nature of islet cell isolation and transplantation. Our center's criteria for transplantation, like many other centers, require an islet dose of approximately 10,000 IEQ/kg

recipient body weight. It has been the experience of our center, as well as other centers, that in order to achieve a transplanted islet cell dose of 10,000 IEQ/kg, multiple islet cell transplants are required from multiple donors. Each transplant introduces a potential multitude of human leukocyte antigen (major histocompatibility complex) (HLA;MHC) mismatches, which, in the context of recipient donor sensitization, reduces the recipients possibility to receive kidney grafts in the future, if necessary. By increasing the effectiveness of the islet cell process and by reducing the cold ischemic organ preservation as demonstrated by our data, fewer islet transplants would be required to attain the completion goal of 10,000 IEQ/kg, thus reducing the recipient’s potential exposure to HLA mismatches. HLA mismatching and its potential role in islet cell graft dysfunction will be the subject of a latter chapter. The efficiency in the utilization of scarce donor organs for islet transplants will also improve by the implementation of shorter cold ischemic organ preservation.

CHAPTER THREE

The Role of Complement in the Destruction of Islet Grafts Post-Transplant

Introduction

Islet transplantation has become an acceptable alternative treatment for patients with type 1 diabetes mellitus and hypoglycemic unawareness. Islet cell transplant graft survival rates have increased following the introduction of the steroid free immunosuppressive regimen at multiple centers[51]; however, long term maintenance of post-transplant insulin independent status is poor[92]. One of the drawbacks of current protocols in islet transplantation is the requirement of multiple donor pancreata to achieve insulin independence. One of the critical components for attaining insulin independence is achieving a large enough engrafted islet mass after transplantation to ensure graft survival[52].

Isolated pancreatic islets are incompatible with human blood, eliciting a host of complications that destroy the transplanted islet mass within the first few hours post-transplant[62]. This incompatibility, known as Instant Blood-Mediated Inflammatory Reaction (IBMIR), involves

coagulation and complement, as well as cellular infiltration. IBMIR research has focused primarily on the outcome of coagulation and lymphocyte infiltration[62], and strategies to prevent the initiation of coagulation[63,93-99], while complement activation has not been studied as extensively[100]. IBMIR elicits platelet and complement activation and deposition on the islet cells, and attracts infiltrating leukocytes to the transplanted islet cells, resulting in entrapment of islet cells within clots[62]. Nilsson et al have demonstrated that pancreatic islet cells contribute to the initiation of IBMIR by expressing tissue factor[63,94,95]. However, little research has been done to analyze the changes in gene expression profile of islets during this process, which in turn could shed light on key molecules and signaling pathways involved in the destruction of pancreatic islet cells during IBMIR.

We previously demonstrated in preliminary work[101] (presented at the 2007 joint conferences of Cell Transplantation Society, International Pancreas and Islet Transplantation Association, International Xenotransplantation Association) that pancreatic islet cells treated with type 1 diabetic serum increased transcription of the cytokines *IL-11* and *12A* and the GTPase Ras Associated with Diabetes (*RAD* aka *RRAD*) while

repressing transcription of the receptor antagonist to IL-1R (*IL-1RN*) that inhibits the harmful effects of inflammatory cytokines such as IL-1B. We hypothesized that complement activation, initiated by autoantibody binding to islet antigens, may effect gene expression changes in islet cells, thus allowing us to predict the key molecules and signaling pathways involved in the destruction of the islet graft. To this end, we further investigated the effect of complement deposition on islet cells using microarray and real-time PCR analysis. Our results indicate that complement contributes to the destruction of the islet graft by inducing expression of IL-1 beta (IL-1β) and other cytokines that attract the cells of both the adaptive immune system and the innate immune system, while concomitantly repressing the transcription of genes that protect against inflammatory cytokines and reactive oxygen species, contributing to the destruction of the islet graft.

Materials and Methods

Pancreas Procurement and Islet Isolation

Donor pancreata were procured through the local organ procurement organization (OPO) after consent for research was obtained. Pancreas removal was performed by Baylor

Regional Transplant Institute (BRTI) surgeons using a standardized whole organ procurement technique to minimize warm ischemia, followed by preservation with the two layer method[102]. Pancreatic islet cells were isolated following the semi automated method described by Ricordi et al[45]. Upon completion of the isolation procedure, the islets were assessed for purity and quantity by staining with dithizone, and viability was assessed by fluorescein diacetate uptake and propidium iodide staining. Islets isolated from five different donors were used.

Serum Treatment of Islet Cells

Freshly isolated islet cells were cultured in islet culture medium containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), that was previously heat inactivated, supplemented with 50mM Trolox C (Sigma Aldrich, St Louis, MO), 2.5mM Niacinamide (Sigma Aldrich, St Louis, MO), and 100 ng/ml insulin growth factor (IGF) (Sigma, St Louis, MO), then placed in 5% CO_2 air at 22°C for twenty-four to forty-eight hours. After culture, islets were counted and viability assessed as described above. Isolated islet cells were divided into three groups. To group I, 1 ml of autologous donor serum was added in triplicate to 1,000 islet equivalents (IEQ). To

group II, 1 ml of ABO matched allogeneic serum from a previous organ donor was added in triplicate to 1,000 IEQ. To group III, 1 ml of serum from patients newly diagnosed with type 1 diabetes (kindly provided by Dr. Ellen Kaizer-Grishman) known to be positive for at least one autoantibody against insulin, glutamic acid decarboxylase 65 (GAD65), or tyrosine phosphatase-like protein (IA-2) was added in triplicate to 1,000 IEQ. Groups I, II, and III from experiments one through four were placed in triplicate in a 24 well plate and incubated at 37°C for three hours. Experiment five was plated similarly, but with six samples per condition. Upon completion of incubation, the islets were removed to separate microfuge tubes and washed with sterile PBS (Gibco, Carlsbad, CA). Islet cells were then placed in RLT lysis buffer (Qiagen, Valencia, CA) containing 0.1% β-mercaptoethanol (β-ME) and stored at -80°C for RNA extraction, or fixed for immunofluorescence imaging and analysis.

Immunofluorescent Imaging

An aliquot of islet cells was taken and fixed in 4% paraformaldehyde (PFA; Sigma, St Louis, MO) in PBS. Fixed islet cells were then centrifuged and placed in Tissue Tek optimal cutting temperature (OCT) compound (Sakura,

Torrance, CA) for cryosectioning. Cryosections were cut at 6μm thickness, and every other section was placed on charged slides beginning with section one. Sixteen sections were taken in total. Sections were then treated with either autologous, allogeneic, or type 1 diabetic serum (obtained from islet cell transplant patients prior to transplant) for two hours for complement fixation at 37°C, and one hour for autoantibody fixation. Sections were then washed in PBS. For autoantibody detection, goat anti-human IgG-FITC (Molecular Probes, Carlsbad, CA) and goat anti-human IgM-FITC (Sigma, St. Louis, MO) were added to separate sections in 1:100 staining buffer (1% FBS:PBS) and incubated at room temperature for 30 minutes. For complement staining, sheep polyclonal anti-human-C3-FITC (Abcam, Cambridge, MA) was added in 1:100 PBS to each section and incubated at room temperature for 30 minutes. Sections were then washed in PBS, followed by a rinse in permeabilization buffer (3% BSA (Sigma, St Louis, MO), 0.1% Triton X-100 (Sigma, St Louis, MO), and 0.1% Azide (Sigma, St. Louis, MO) in PBS). Sections were then placed in the permeabilization buffer for 15 minutes at room temperature. Mouse monoclonal anti-insulin (Abcam, Cambridge, MA) conjugated to AF647 (Invitrogen, Carlsbad, CA) was diluted 1:100 in permeabilization buffer and added to each section

for 30 minutes at room temperature for the purpose of islet identification. Sections were washed twice in permeabilization buffer, followed by a third rinse in PBS. DAPI (Invitrogen, Carlsbad, CA) was added to each section in a 1:5000 dilution in PBS and incubated at room temperature for five minutes. Sections were washed twice in PBS, dried briefly, and covered with Fluoromount-G (Southern Biotech, Birmingham, AB) before coverslipping. All incubations with fluorescently labeled antibodies were done in the dark. Goat anti IgG-FITC (Caltag, Carlsbad, CA), sheep IgG (Abcam, Cambridge, MA) conjugated to FITC (Sigma, St Louis, MO) and mouse IgG1 (R&D Systems, Minneapolis, MN) conjugated to AF647 (Invitrogen, Carlsbad, CA) served as isotype controls. Images were taken on an upright fluorescent microscope using MetaMorph 6.2r6 (Molecular Devices, Sunnyvale, CA) for image acquisition and analysis.

Microarray Analysis

Total RNA was isolated using TRI Reagent (Ambion, Austin, TX) following the manufacturer's instructions for experiments one to three. For experiments four and five, total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions.

Coding mRNA from total RNA was used to generate double stranded cDNA by reverse transcription. Double stranded cDNA served as a template for the production of biotinylated cRNA using the Illumina Total Prep mRNA Isolation kit (Ambion, Austin, TX). Biotinylated cRNA was hybridized to Illumina human whole genome Hu6 V2 or V3 (experiment 4) gene chips (Illumina, San Diego, CA) following the manufacturer's directions. Hybridized gene chips were then scanned on an Illumina BeadStation 500 following the manufacturer's protocols (Illumina, San Diego, CA). Illumina's BeadStudio software was used to generate signal intensity values from the scans, subtract background, and scale each microarray to the median average intensity for all samples (per-chip normalization). Data analysis was performed using GeneSpring GX 7.3 software (Agilent, Santa Clara, CA). Samples were clustered independently to determine if gene expression reflected treatment condition. Upon completion of unsupervised clustering analysis, experiment five was chosen as a representative experiment for further class prediction analysis. The software generated a list of 50 genes that predicted gene condition 100% correctly in experiment five (training set). This list of genes was then tested against the other experiments (test sets). From this list,

significant gene expression changes as determined by one-way ANOVA analysis were chosen for further analysis among all experiments.

Real Time PCR

One representative sample from each condition in each experiment was randomly chosen for confirmation of the data obtained in microarray analysis. Total RNA was reverse transcribed to generate cDNA using RetroScript (Ambion, Austin, TX) cDNA synthesis kit. 100 ng of cDNA was plated in triplicate for each condition and each gene of interest. TaqMan (Applied Biosystems, Foster City, CA) probes specific *MMP-9, IL-11, IL-12A, RAD, IL-1RN, IL-1*β were used to specifically amplify these genes of interest. *GAPDH* served as a normalizing housekeeping gene. Relative quantity of the gene of interest (GOI) was compared to the autologous (calibrator) condition for both allogeneic and diabetic conditions by the $2^{\Delta\Delta Ct}$ method for relative quantification. Replicates were averaged from each experimental condition for each gene. Each experiment was then averaged for each gene of interest.

Statistical Methods

All data is expressed as mean ±SEM. Grubbs method of detecting statistical outliers was used to remove outliers

from further statistical analysis. One-way ANOVA non-parametric T testing with multiple error correction was used to determine statistical significance of the micro-array data, with statistical significance being $p<0.01$. For real-time PCR analysis, students two-way unpaired t-test was used to determine significance. Results were considered significant when $p<0.05$.

Results

Complement Activation by Treatment of Islets with Type 1 Diabetic Serum

Following intraportal infusion, pancreatic islet cells undergo IBMIR, which consists of activation of both coagulation and complement cascades. To investigate the role complement plays in the process of graft destruction that begins immediately post-transplant, we analyzed the gene expression changes that pancreatic islet cells undergo when exposed to complement. To this end, type 1 diabetic serum obtained from patients prior to islet transplant was used to treat sectioned islet cells. To establish that autoantibodies present in diabetic serum are responsible for the fixation of complement upon isolated islet cells, we stained serum treated islet cells for the presence of IgG and IgM (Figure 3.1). Autoantibodies were only

detected in the diabetic serum condition when compared to the autologous and allogeneic condition. To establish that autoantibodies are capable of fixing complement to the surface of islet cells, we next stained serum treated sections for the presence of complement factor C3. The

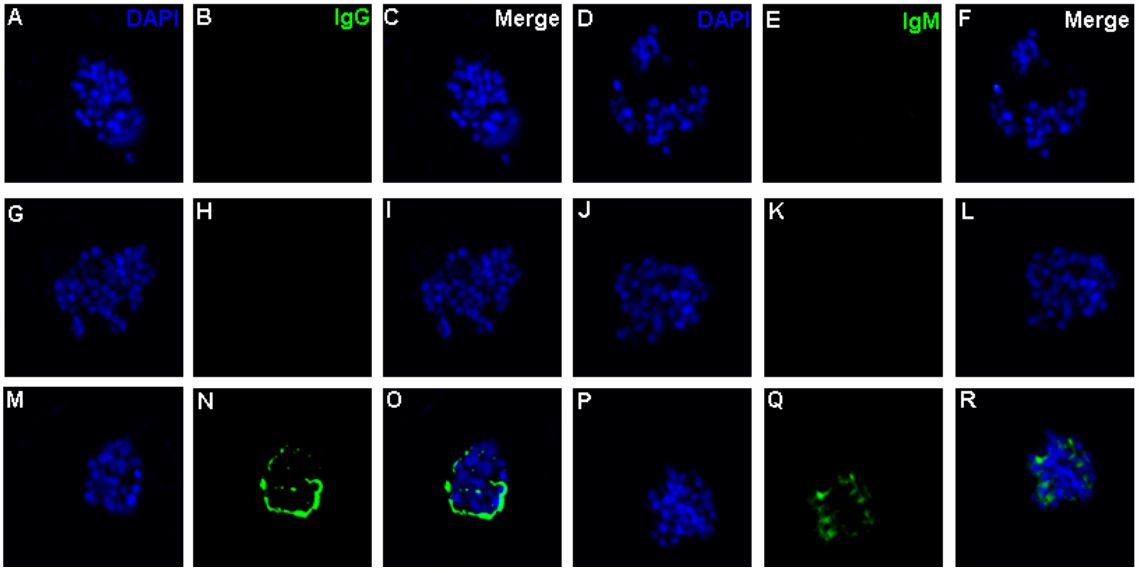

Figure 3.1 Binding of Auto-Antibodies From Diabetic Serum to Islet Cells. Isolated islet cells were fixed and cryosectioned at 6μm intervals. Islet cell sections were then incubated in a 1:2 dilution of autologous serum (A-F), allogeneic serum (G-L), and type 1 diabetic serum (M-R) at 37°C for 1 hour. Sections were washed and labeled with goat anti-human IgG-AF488 or goat anti-human IgM-FITC. Only type 1 diabetic serum treated islet sections demonstrated the presence of autoantibodies as detected by the presence of IgG (N) and IgM (Q).

sectioned islet cells were then stained with an antibody for complement factor C3 (Figure 3.2). Islet sections that were exposed to type 1 diabetic serum alone demonstrated the presence of complement (Figure 3.2K), while those sections that were exposed to autologous or non-diabetic allogeneic serum did not show any complement deposition.

These data indicate that autoantibodies present in type 1 diabetic serum are capable of recognizing islet antigens, which initiates the deposition of complement on the

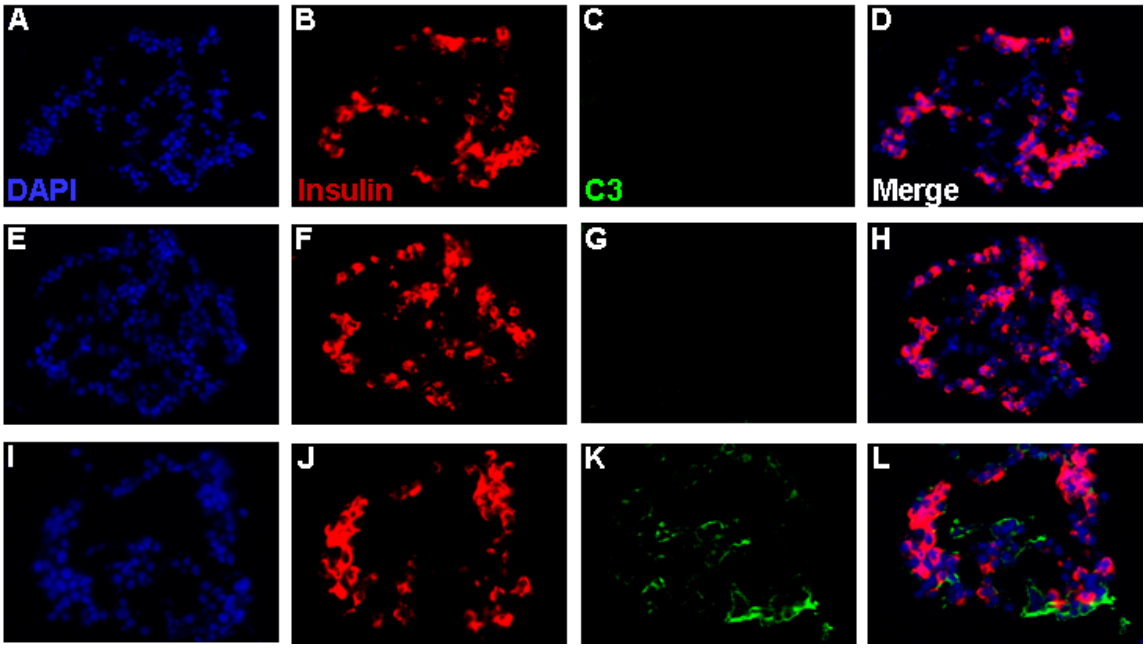

Figure 3.2 Complement Deposited on Islets Exposed to Type 1 Diabetic Serum. Isolated islet cells were fixed and cryosectioned at 6um intervals, four consecutive odd numbered sections were used per condition. Sections 7, 15, and 23 shown. Islet sections were then incubated in a 1:2 dilution of autologous serum (A-D), allogeneic serum (E-H) and type 1 diabetic serum (I-L) at 37°C for 2 hours. Sections were washed and labeled with rabbit polyclonal anti-C3-FITC and mouse monoclonal anti-insulin conjugated to AF647. Only type 1 diabetic serum displayed signs of complement deposition (K), which corresponded with an islet cell represented by insulin (J).

surface of the islet cell. Because autologous and allogeneic donor serum does not contain islet cell autoantibodies, there is no evidence of complement deposition on the surface of islet cells (Figure 3.2C,G).

Complement Induces Gene Expression Changes in Isolated Islets

Isolated islet cells were treated with diabetic serum known to be autoantibody positive to further investigate gene expression changes induced by autoantibody and complement activation. Gene profiles that lacked any detectable signal intensity across the board were removed from analysis in an attempt to reduce false discovery. Flat expression profiles were removed from data analysis by filtering the data on the criteria of meeting the minimum fluorescence cutoff and having a two fold difference in expression in at least one sample when compared to the mean of the autologous condition for each gene. This dramatically reduced the number of genes from ~48,000 genes to an average of 3,415 ± 1,552 genes. Supervised clustering performed on the filtered microarray data demonstrated that the samples clustered by treatment condition (Figure 3.3). This demonstrates the difference is gene expression between the three treatment groups. Because of the different gene expression due to complement fixation brought on by autoantibody recognition of islet cells, we set out to determine if there were any genes that could be used as possible intervention targets. To this end class prediction analysis was then performed in an

attempt to compile a list of genes that could be attributed to islet autoantibodies and complement deposition (Figure 3.4). A representative experiment was used as the training set to establish whether the predictor genes could predict

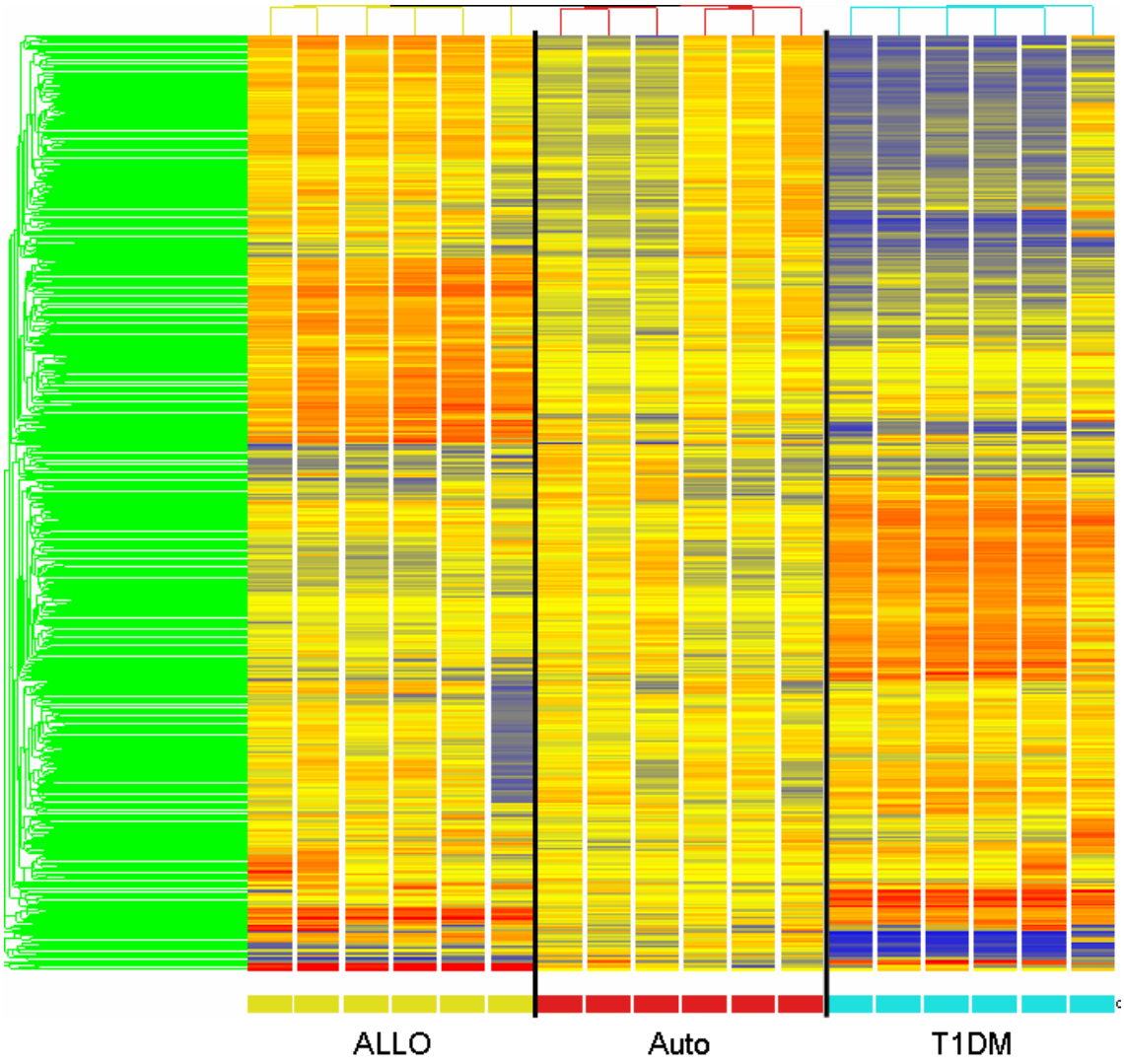

Figure 3.3 Complement Activation Initiated by Autoantibodies in Type 1 Diabetic Serum Induce a Specific Profile of Gene Expression. Islet cells exposed to complement in diabetic serum undergo changes in gene expression that leads to the self-destruction of the islet graft. Unsupervised clustering reveals 679 genes that are differentially expressed in diabetic serum when compared to autologous serum. Data from a representative experiment is shown.

the correct treatment classification in itself. Once the predictor list was established as having 100% correct prediction in the training set, this list was imported to

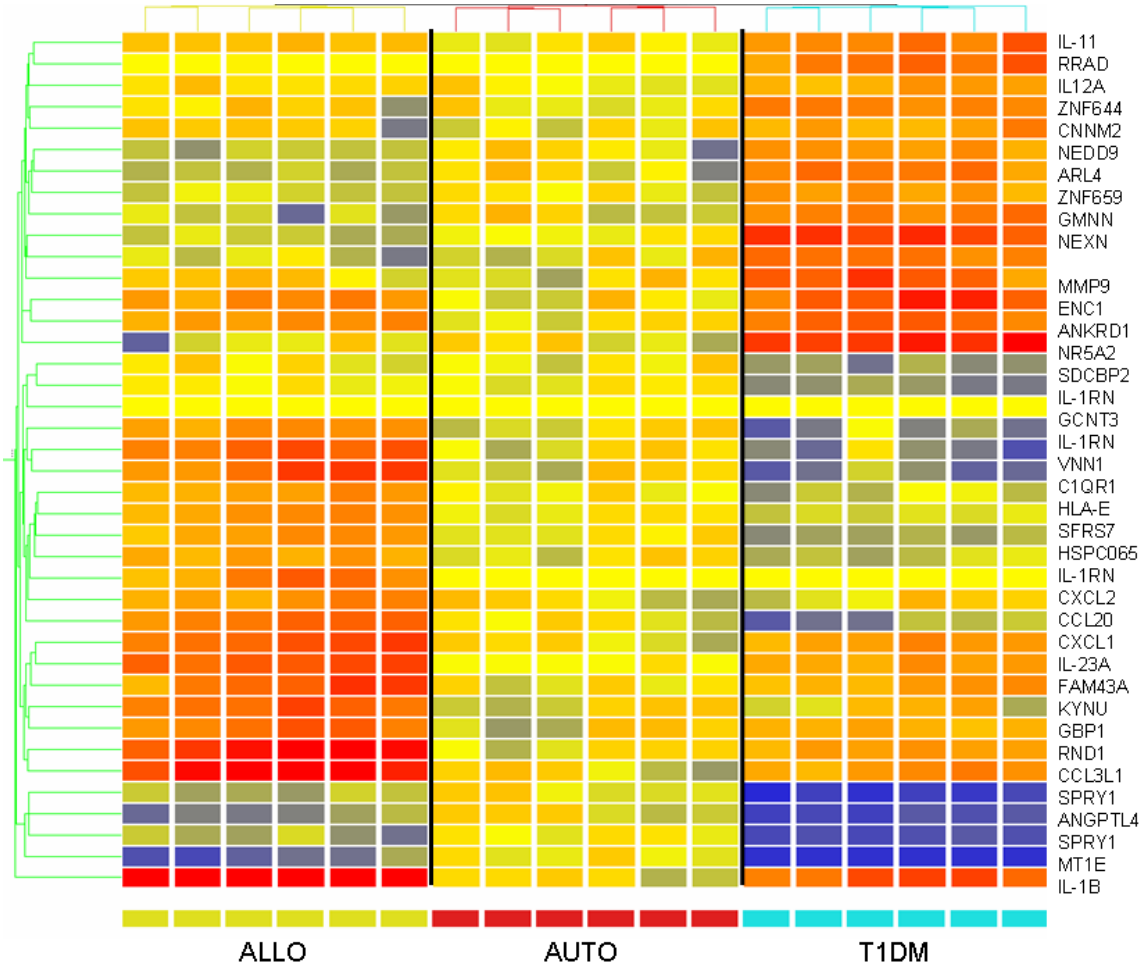

Figure 3.4 Class Prediction Analysis of Islets Exposed to Diabetic Serum Based on Changes in Genetic Profile. A representative experiment was chosen for class prediction analysis to establish a list of genes that could differentiate the serum conditions based on gene expression changes. Samples were normalized to the mean of the autologous condition.

the other experiments for further analysis, resulting in a mean prediction accuracy of 56% ± 17%. The predictor genes were analyzed for the expression profile for each

experiment and the resulting data compiled for an overall expression pattern (Table 3.1). This table implies that type 1 diabetic serum induces significant gene expression changes through complement activation. When compared to normal autologous expression, the following genes were significantly over-expressed: RRAD (343.17 ± 62.37, p=0.0008), GMNN (284.59 ± 40.54, p<0.0001), ANKRD1 (279.99 ± 45.87, p=0.0003), IL-1β (255.66 ± 31.31, p<0.0001), IL-23A (251.37 ± 35.85, p=0.0002), MMP9 (243.37 ± 35.13, p=0.0001), IL-11 (220.12 ± 45.46, p=0.0135), ENC1 (219.3 ± 15.27, p<0.0001), SDS (210.56 ± 26.46, p=0.0004), GBP1 (181.43 ± 30.03, p<0.0001), NEXN (179.03 ± 15.65, p<0.0001), EPC1 (175.61 ± 16.43, p=0.0001), CXCL1 (146.62 ± 19.66, p=0.0256); while the following genes were significantly under-expressed: SDCBP2 (66.73 ± 8.78, p=0.004), HSPC065 (64.27 ± 10.42, p=0.0243), IL-1RN (64 ± 6.02, p=0.0049), CCL20 (61.86 ± 8.36, p=0.0008), ANGPTL4(48.1 ± 7.76, p<0.0001), SPRY1 (37.13 ± 6.24, p<0.0001), MT1E (34.48 ± 10.71, p<0.0001).

Real-time QPCR of Select Genes Corroborates Changes Indicated by Micro-Array Analysis

In an attempt to substantiate the data collected by micro-array analysis, representative genes were chosen for

further analysis by real-time quantitative PCR. TaqMan probes specific for matrix metalloproteinase -9 (*MMP-9*),

Table 3.1 Average Signal Intensity for Class Predictor Genes

	Experiment						
Gene ID	1	2	3	4	5	Average	p
MMP9*	62.98 ± 8.32	181.58 ± 24.99	4682.43 ± 228.46	209.49 ± 23.54	351.34 ± 47.45	243.37 ± 35.13	0.0001
KYNU	49.12 ± 11.13	75.84 ± 22.11	2918.49 ± 49.56	204.52 ± 22.01	114.78 ± 16.7	116.29 ± 16.86	0.3803
IL-1B*	70.49 ± 4.26	170.88 ± 31.75	1656.21 ± 57.90	363.32 ± 31.84	305.95 ± 29.58	255.66 ± 31.31	<0.0001
GBP1	110.54 ± 12.08	259.00 ± 3.67	881.78 ± 162.58	247.02 ± 11.43	146.4 ± 5.14	181.43 ± 16.84	<0.0001
SDS	136.18 ± 1.19	343.99 ± 0.51	22.7 ± 2.50	249.79 ± 33.42	233.89 ± 22.49	210.56 ± 26.46	0.0004
IL11*	84.23 ± 11.95	523.35 ± 81.56	1.81 ± 1.81	120.58 ± 7.14	236.35 ± 23.16	220.12 ± 45.46	0.0135
ANKRD1	101.59 ± 3.99	297.89 ± 48.18	1.47 ± 1.47	205.48 ± 28.09	460.61 ± 42.51	279.99 ± 45.87	0.0003
GMNN	120.57 ± 3.84	275.70 ± 1.77	5.58 ± 0.92	423.90 ± 11.9	383.70 ± 24.65	284.59 ± 40.54	<0.0001
ENC1	111.48 ± 5.62	240.93 ± 17.51	186.86 ± 0.74	191.41 ± 9.80	269.18 ± 17.81	219.30 ± 15.27	<0.0001
HLA-E	94.87 ± 5.29	127.75 ± 11.58	778.72 ± 197.71	120.00 ± 6.44	84.01 ± 2.54	187.16 ± 32.35	0.2664
CCL4L1	65.10 ± 6.04	74.04 ± 15.65	1187.75 ± 234.24	116.52 ± 12.24	206.97 ± 16.64	266.89 ± 57.03	0.1038
IL-23A	116.04 ± 7.01	484.44 ± 51.86	345.51 ± 70.15	117.38 ± 3.57	215.56 ± 10.90	251.37 ± 35.85	0.0002
STAT5A	89.07 ± 1.47	114.92 ± 0.12	754.27 ± 158.85	152.59 ± 10.87	85.85 ± 5.22	187.03 ± 35.81	0.2182
ZNF659	89.84 ± 9.47	139.77 ± 3.11	443.96 ± 117.27	106.61 ± 11.31	223.56 ± 12.19	183.94 ± 30.03	0.0049
NEXN	100.65 ± 0.34	138.07 ± 22.97	112.47 ± 9.44	188.57 ± 10.67	243.07 ± 11.14	179.03 ± 15.65	<0.0001
FAM43A	85.43 ± 0.09	126.29 ± 7.92	551.7 ± 54.59	107.44 ± 1.73	163.84 ± 13.61	184.90 ± 37.17	0.0263
RRAD*	117.55 ± 3.43	249.98 ± 0.43	1.58 ± 1.58	192.41 ± 0.45	543.75 ± 52.04	343.17 ± 62.37	0.0008
C1QR1	91.75 ± 4.87	68.97 ± 9.7	717.43 ± 95.04	80.13 ± 4.65	64.63 ± 2.43	158.63 ± 36.78	0.7955
IL-18R1	74.61 ± 9.40	103.96 ± 1.64	532.48 ± 45.22	89.72 ± 6.48	57.78 ± 7.02	136.37 ± 30.01	0.8777
EPC1	92.65 ± 11.04	159.00 ± 21.85	264.45 ± 41.14	103.66 ± 2.43	217.93 ± 7.38	175.61 ± 16.43	0.0001
NCOA7	96.79 ± 4.63	92.98 ± 1.30	54.20 ± 0.87	121.94 ± 4.79	226.27 ± 16.4	145.57 ± 19.01	0.0216
ZNF658	15.28 ± 15.28	82.13 ± 20.49	142.82 ± 18.51	44.78 ± 10.11	213.5 ± 4.54	129.53 ± 20.99	0.2211
IL-12A*	123.88 ± 0.71	158.01 ± 14.5	26.41 ± 8.35	97.91 ± 11.85	176.46 ± 10.04	132.94 ± 13.70	0.0578
CXCL1	92.42 ± 2.48	127.80 ± 0.24	35.56 ± 4.63	113.63 ± 3.43	226.27 ± 16.4	146.62 ± 19.66	0.0256
IL-1RN*	76.97 ± 9.33	61.08 ± 11.03	975.22 ± 41.11	165.91 ± 2.59	61.14 ± 9.44	64.00 ± 6.02	0.0049
ZNF644	121.48 ± 4.32	168.74 ± 3.20	88.24 ± 3.14	108.43 ± 0.51	167.68 ± 15.83	130.73 ± 10.43	0.0016
NEDD9	103.9 ± 7.95	121.35 ± 7.59	63.69 ± 36.49	92.59 ± 0.60	226.91 ± 16.7	137.78 ± 18.92	0.0161
1780523	94.14 ± 1.50	118.01 ± 3.00	82.25 ± 13.91	N/A	299.64 ± 35.17	192.66 ± 32.69	0.0100
CNNM2	108.26 ± 7.84	100.72 ± 11.43	4.40 ± 2.20	89.80 ± 29.17	179.97 ± 8.04	119.42 ± 15.84	0.3470
RND1	121.21 ± 0.04	86.97 ± 4.49	0.00 ± 0.00	101.49 ± 8.64	166.53 ± 6.94	117.11 ± 14.88	0.3477
ARL4	108.63 ± 8.02	108.75 ± 0.28	108.40 ± 9.27	92.87 ± 6.54	180.33 ± 11.79	131.02 ± 11.24	0.0083
SLC25A3	127.67 ± 37.02	112.23 ± 6.82	39.13 ± 17.39	102.31 ± 2.61	192.29 ± 15.89	133.19 ± 15.01	0.0434
CXCL2	100.71 ± 0.55	143.64 ± 0.59	0.00 ± 0.00	92.20 ± 2.54	107.40 ± 10.55	68.26 ± 11.37	0.6288
LAMB3	69.92 ± 6.86	69.18 ± 2.30	0.00 ± 0.00	140.74 ± 6.06	183.40 ± 15.97	33.93 ± 18.56	0.3128
CCL3	81.76 ± 8.69	141.6 ± 0.28	16.71 ± 8.88	68.94 ± 5.02	196.30 ± 13.29	122.87 ± 18.69	0.2104
CCL3L1	91.20 ± 6.85	93.67 ± 15.64	20.94 ± 0.07	66.21 ± 4.93	183.40 ± 15.97	112.77 ± 16.41	0.4620
CCL3L3	96.35 ± 7.91	143.54 ± 0.28	13.21 ± 4.99	64.63 ± 6.18	187.02 ± 15.04	120.05 ± 18.04	0.2372
SLC25A36	95.41 ± 1.63	109.42 ± 26.16	15.42 ± 4.18	77.52 ± 9.53	139.68 ± 4.45	101.29 ± 11.33	0.9154
GCNT3	95.75 ± 2.80	85.88 ± 5.09	0.35 ± 0.35	148.04 ± 27.44	56.90 ± 9.22	79.71 ± 13.39	0.1150
NR1D2	105.15 ± 2.80	119.89 ± 6.80	37.93 ± 3.50	75.56 ± 6.69	153.50 ± 3.79	97.71 ± 10.52	0.3283
TLCD1	100.49 ± 0.72	103.64 ± 3.08	19.07 ± 0.14	106.36 ± 2.18	86.18 ± 4.79	85.71 ± 7.80	0.0828
SFRS7	98.00 ± 3.50	67.81 ± 6.20	165.32 ± 2.78	141.98 ± 3.60	64.63 ± 2.43	96.49 ± 10.16	0.7461
SERPINB2	95.29 ± 0.63	88.03 ± 0.48	7.52 ± 1.65	67.73 ± 6.91	183.40 ± 15.97	109.23 ± 17.98	0.5174
HSPC065	93.37 ± 18.69	100.97 ± 8.84	0.00 ± 0.00	72.95 ± 41.61	76.24 ± 4.55	64.27 ± 10.42	0.0243
MT1E	93.65 ± 3.40	13.95 ± 0.29	7.26 ± 7.26	90.17 ± 1.32	4.78 ± 1.10	34.48 ± 10.71	<0.0001
CCL20	85.95 ± 7.76	66.00 ± 0.36	2.20 ± 0.39	99.18 ± 6.37	58.82 ± 7.83	61.86 ± 8.36	0.0008
VNN1	98.59 ± 2.57	40.82 ± 5.42	1255.88 ± 55.18	142.96 ± 28.75	48.03 ± 7.96	148.68 ± 108.07	0.2311
NR5A2	77.63 ± 0.97	119.89 ± 6.80	0.04 ± 0.04	193.00 ± 2.41	58.91 ± 3.58	83.99 ± 14.84	0.2859
ARRDC3	87.79 ± 2.24	41.68 ± 8.85	161.7 ± 32.65	102.79 ± 2.35	47.55 ± 2.58	72.90 ± 11.45	0.0576
SDCBP2	82.86 ± 6.95	91.46 ± 4.69	1.18 ± 0.12	111.67 ± 0.66	55.85 ± 3.03	66.73 ± 8.78	0.0040
SPRY1	89.65 ± 1.18	45.47 ± 0.02	8.59 ± 0.15	38.65 ± 1.66	25.6 ± 1.55	37.13 ± 6.24	<0.0001
ANGPTL4	71.01 ± 4.51	22.13 ± 6.47	0.00 ± 0.00	75.36 ± 24.49	55.85 ± 3.03	48.10 ± 7.76	<0.0001

*indicates gene expression corroborated with Real-time PCR

interleukin 1-beta (*IL-1β*), interleukin 11 (*IL-11*), interleukin 12A (*IL-12A*), ras-related associated with

diabetes (*RAD*), and interleukin 1 receptor antagonist (*IL-1RN*) were used to amplify these genes. Due to monetary reasons, a representative sample from each condition was chosen from each experiment, totaling 15 samples. Total RNA left over from the micro-array analysis was used to generate cDNA. Relative quantification of the T1DM condition, with respect to the autologous condition, confirmed the respective change in gene expression (Figure 3.5). *MMP-9* demonstrated a 7.50 fold up-regulation in T1DM when compared to autologous serum treatment. *IL-1β* likewise depicted a significant 2.23 fold up-regulation ($p<0.01$) in the T1DM condition. Although not significant, *IL-11* was also up-regulated by 1.92 fold in the diabetic condition. *IL-12A* underwent a significant 3.19 fold up-regulation when treated with diabetic serum ($p<0.05$). *RAD* expression was significantly up-regulated by 6.13 fold by T1DM serum ($p<0.05$) when compared to autologous serum. The expression of *IL-1RN* in T1DM treated islets was significantly reduced to 0.42 of the autologous expression level ($p<0.01$).

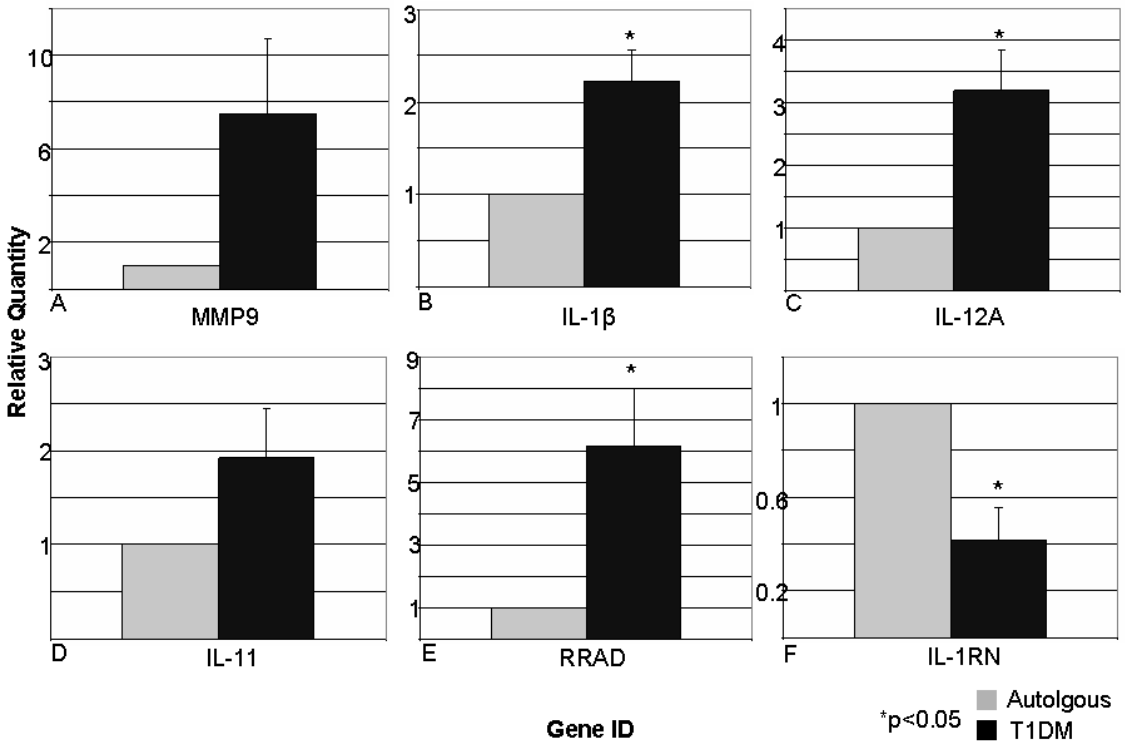

Figure 3.5 Real Time PCR Analysis Confirms Micro-Array Data in Representative Genes. Representative genes were chosen for corroboration by using relative quantification real-time PCR. TaqMan probes specific for MMP-9 (A), IL-1β (B), IL-12A (C), IL-11 (D), RRAD (E), and IL-1RN (F) were used to produce cDNA from representative mRNA samples from each experiment. Real-time PCR detected significant gene expression changes of 7.50 ± 3.17 (p=0.07), 2.23 ± 0.33 (p=0.005), 3.19 ± 0.66 (p=0.01), 1.92 ± 0.53 (p=0.12), 6.13 ± 1.87 (p=0.03), 0.42 ± 0.14 (p=0.003) respectively. Mean ± SEM.

Discussion

Instant Blood-Mediated Inflammatory Reaction (IBMIR) is known to involve both the coagulation cascade and the complement cascade[62,99,103]. In the present study, we investigated the molecular mechanism(s) by which complement activation contributes to the destruction of the islet cell graft. Pancreatic islets are isolated mostly from brain dead donors experiencing cytokine storm[104]. Hence, donor

serum was used as a control to establish the background effects of cytokines on isolated islet cells. Similarly, ABO matched allogeneic donor serum was also used to control for mismatch in HLA compatibility. Sera from a living type 1 diabetic patient known to be positive for islet auto-antibodies was used to simulate islet transplantation[105]. The resulting gene expression profile data reflects the conditions of clinically transplantable islet cells undergoing IBMIR due to the soluble complement factors. We can eliminate the contribution of coagulation in these changes because serum was used for the treatment of islets rather than plasma.

Micro-array analysis generated an average list of genes (3,415 ± 1552) that were differentially expressed between the three conditions in the five experiments analyzed. In an attempt to reduce the amount of data to a workable level, class prediction analysis was implemented using a representative experiment. The resulting data set of 50 genes was now easily manageable. The list of predictor genes generated from experiment five was imported to the analysis of the remaining experiments for expression analysis. The expression values for each gene for each sample from each experiment were exported to excel, and the samples were averaged. The resulting data demonstrated a

trend of increasing transcription of genes that have activity toward angiogenesis while turning off transcription of genes which protect the islets from inflammatory cytokines and reactive oxygen species.

Matrix Metalloproteinase-9 (MMP-9) is an enzyme that plays a pivotal role in the migration of immune cells[106]. MMPs have even been demonstrated to be present on rodent islets after isolation[107]. It has been shown to promote angiogenesis in tumor cells by proteolytically cleaving latent TGF-β into active TGF-β[108]. A previous study using a mouse model of islet transplantation has demonstrated that pre-treatment of islets with MMP-9 improved islet graft revascularization, with improved vascular density, blood flow and oxygen tension[109]. It is plausible that islet cells up-regulate transcription of MMP-9 in an attempt to initiate angiogenesis to improve their oxygen supply.

Interleukin 1 beta (IL-1β) is an inflammatory cytokine primarily involved in inflammation and immune responses[110]. In tumor models, IL-1β has been shown to promote tumor angiogenesis, while IL-1RN was demonstrated to block angiogenesis[110]. IL-1β has also been demonstrated to cause the nuclear exportation of N-CoR corepressor complex, resulting in the depression of NF-$_{\kappa}$B regulated genes[111]. It is possible that islets transplanted into the hepatic

portal vein, in an attempt to promote revascularization, due to a hypoxic environment, up-regulate IL-1β along with other pro-angiogenesis while inhibiting transcription of blockers of angiogenesis (IL-1RN) factors to promote revascularization of the islet graft. However, due to the inflammatory environment that the islets encounter, the amount of IL-1β may be beyond levels required for angiogenesis and adequate for inflammation and apoptosis. Indeed, it has been demonstrated that IL-1β induces NF-$_{\kappa}$B leading to the downstream activation of genes that promote beta cell dysfunction and apoptosis[112], and IL-1β has been demonstrated to be involved in the onset of autoimmune diabetes[113]. This would be exacerbated in that islets are inhibiting the transcription of the receptor antagonist (IL-1RN) that blocks IL-1β-IL-1R signaling.

Interleukin 11 is known to stimulate T cell dependent B cell development as well as plasmacytoma proliferation[114]. In addition to IL-11's hematopoietic regulatory function, IL-11 has also been demonstrated to induce synthesis of the tissue inhibitor of metalloproteinase-1 (TIMP-1), preventing the breakdown of extracellular matrix[115]. IL-11 is also a stimulator of megakaryocyte development and proliferation leading to increased platelet counts in murine and non-human primate[115] models. Some non-immune

cells, human articular chondrocytes[116], have been shown to express IL-11 mRNA upon stimulation with IL-1 or TGF-β, creating the possibility that pancreatic islet cells could be induced to express IL-11. The expression of IL-11 could lead to the development of B cells sensitized to the islet graft, hindering the long term engraftment of the islet cells.

Interestingly, IL-12A (IL-12p35) and IL-23A (IL-23p19), which both share a common IL-12p40 subunit, were both up-regulated by diabetic serum in islet cells. When the p35 and p19 subunits couple together with p40, they form IL-12 and IL-23 respectively. These two cytokines have opposite effects, with IL-12 promoting cell mediated immunity[117] and IL-23 promoting memory T cells, regulatory T cells, angiogenesis and MMP-9 activity[117,118]. IL-12 has also been demonstrated to possess the ability to suppress apoptosis induced by UV radiation[119] and aids IL-2 in activating tonsilar and lymph node NK cells[120]. In addition, Epstein-Barr virus has been shown to induce a gene (*EBI3*) that is similar to the IL-12p40 subunit, enabling the formation of the IL-35 cytokine[121]. IL-35 has been demonstrated to promote the proliferation of regulatory T cells, while inhibiting the proliferation of Th17 cells[121,122].

Ras associated with diabetes (RAD), a member of the Ras family of small GTPases, has been reported to be over-expressed in muscle tissue of patients diagnosed with type 2 diabetes mellitus[123]. Ras is normally involved in signal transduction through many pathways including the MAP Kinase pathway. Ras is known to control such cellular mechanisms as proliferation, differentiation and apoptosis[124,125]. Transcription of the chemokine CXCL1 has been demonstrated to be turned on by Ras activation[126]. CXCL1 has been reported to be involved in angiogenesis[127].

Metallothionein IE (MT1E) is ubiquitously expressed and is known to function in the sequestering of heavy metal atoms within the cell, detoxify metals, regulate the homeostasis of zinc and copper during development, regulate synthesis and activity of zinc metalloproteins, and provide protection against reactive oxygen species[128]. Production of ROS has been reported to play a fundamental role in pancreatic beta cell death in islet transplantation[129]. Down-regulation of MT1E would make the pancreatic beta cells even more sensitive to ROS-mediated cell death.

IL-1 receptor antagonist (IL-1RN) has been demonstrated to be down-regulated in patients with T2DM[130]. In vitro studies have shown that reduced expression of IL-

1RN coupled with increased expression of IL-1β lead to impaired beta cell function and increased activation of apoptosis[130]. Our data indicates that serum from T1DM patients has the ability to down-regulate IL-1RN in vitro, indicating that a similar phenomenon may be occurring during islet cell transplantation. This would be detrimental to the survival of the transplanted islet mass, as IL-1β is produced during post-transplant inflammation. A recombinant form IL-1RN, used as a treatment for T2DM, has shown some recent promise by improving glycemic control and beta cell secretory function[131].

Taken together, the gene array data indicate that the islet cells are trying to overcome hypoxic conditions experienced during the immediate post-transplant period by inducing or inhibiting transcription of genes that can promote/inhibit angiogenesis. Alternatively, while trying to compensate for the hypoxic conditions encountered in the hepatic sinusoids, the islets make themselves vulnerable to immune attack by attracting innate immune cells, reducing protection against inflammatory cytokines, and reducing their ability to combat reactive oxygen species. The results of this study demonstrate areas of possible intervention, mainly through antagonists to IL-1β. Future studies could be aimed at elucidating what protective role

IL-1β blockers such as Anakinra could have on the transcriptional profile of islet cells during the immediate post-transplant period.

CHAPTER FOUR

The Expression of HLA Class II Molecules on the Surface of Isolated Islet Cells and their Role in Sensitization and Rejection[a]

Introduction

Clinical islet transplantation is a promising treatment for “brittle” type 1 diabetic patients to achieve normoglycemia[46]. Following the publication of Edmonton protocol[46], achievement of insulin independence was considered as a primary goal of islet transplantation and several experienced centers were able to successfully reproduce short term results shown by the Edmonton group[51]. However, the long term insulin independent rates have dropped from 80% insulin independence at 1 year to about 8% at five years[92]. The mechanisms for the loss of islet function are not clearly understood. There are several possible causes for islet allograft loss, such as islet quality, Instant Blood Mediated Inflammatory Reaction (IBMIR), adverse effects of immunosuppressive drugs, and the development of immune response against the donor islets[62,76].

[a] Adapted with permission from Jackson A, et al[132]. Copyright 2009, Society of Transplant Surgeons.

Currently, islet transplantation does not require performing HLA matching between the donors and recipients. Furthermore, multiple infusions of islets isolated from different donor organs are generally required to reach adequate transplanted islet mass to attain insulin independence. Such multiple islet infusions increase the number of possible mismatched HLA antigens[77]. Not surprisingly, several reports have shown that islet transplant recipients develop antibodies to the transplanted donor HLA antigens, particularly following withdrawal of immunosuppression[77-81]. Detailed analyses have shown that the islet transplant recipients develop antibodies to both HLA class I and II antigens of the donor origin (Table 4.1). While it is evident that islets constitutively express HLA class I antigens, the expression of HLA class II on isolated human islets has not been clearly demonstrated. Upon stimulation with TNFα and IFNγ, islet-enriched pancreatic cultures[84,133] and MIN 6[85] cell lines have been shown to express HLA class II antigens. However, such demonstration in isolated human islets is still lacking. In addition, the mechanism of HLA sensitization in islet allograft recipients remains unclear. We hypothesized that under inflammatory conditions, there is induction of HLA class II expression

on the islet cell surface leading to the development of anti-donor class II antibodies.

Table 4.1 HLA Sensitization in Islet Transplant Recipients

Center	# of Patients	anti-class I	anti-class II
University of Virginia[i]	1	class I	none
Washington University[ii]	3	class I	none
University of Pennsylvania[iii]	1	class I	class II
University of Alberta[iv]	14	class I	class II
University of Minnesota[v]	4	class I	class II
BRTI[vi]	1	none	DR7
BRTI	1	A1, A23, A24, A32, A36, B38, B49, B51, B53, B59, B63, B77	DR1, DQ7, DQ8, DQ9

i. Lobo PI, et al. Transplant Proc. 2005; 37: 3438.
ii. Mohanakumar T, et al. Transplantation. 2006; 82: 180
iii. Rickels MR, et al. J Clin Endocrinol Metab. 2007; 92: 2410.
iv. Campbell PM, et al. Am J Transplant. 2007; 7:2311.
v. Bellin MD, et al. Am J Transplant. 2008; 8: 2463.
vi. Unpublished data

HLA molecules are critical to the immune response. HLA molecules are encoded within the major histocompatibility complex (MHC) region on chromosome 6. There are two classes of HLA molecules, each with several subclasses. They are both surface molecules composed of four subunits and a transmembrane domain (Figure 4.1). They share a similar function in that they both present peptides to lymphocytes. However, they differ in that HLA class I molecules are present on all cells of the human body, and are responsible for presenting peptides of primarily

intracellular origin, such as cytosolic proteins generated by intracellular viruses, to cytotoxic effector T cells. HLA class II molecules are primarily restricted to antigen

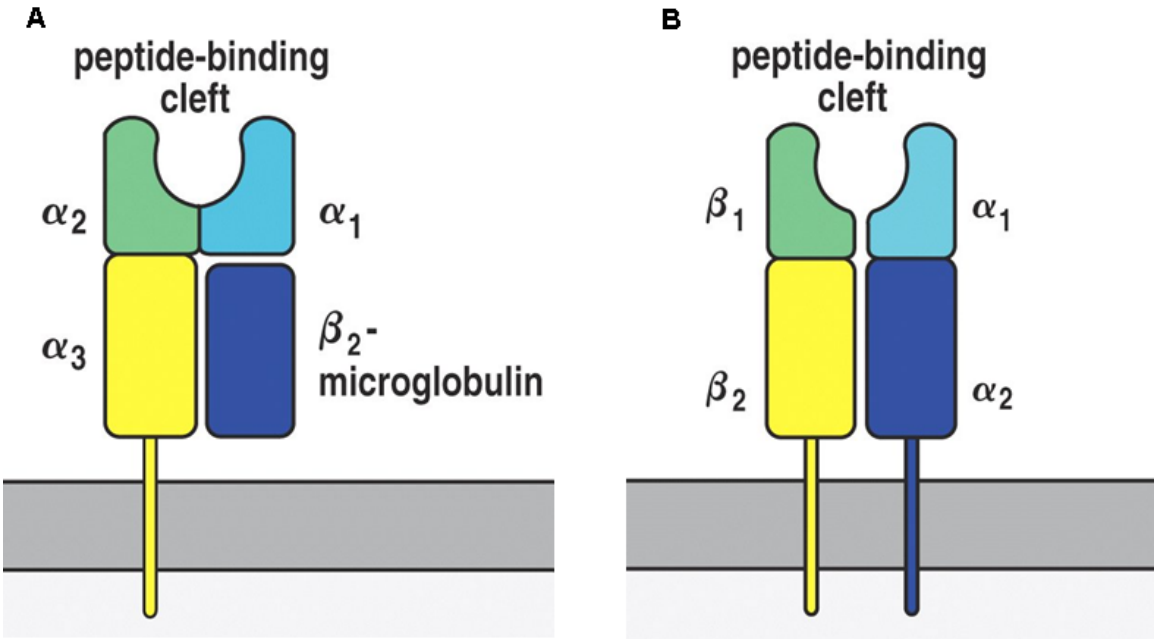

Figure 4.1 Structure of the Human Leukocyte Antigen (HLA). HLA class I (A) is composed of a heavy chain containing three alpha subunits (α1, α2 , α3) and a non-HLA protein, β2 microglobulin. There are three major classes of HLA class I, designated HLA-A, HLA-B, HLA-C. HLA class II (B) is composed of two polypeptides chains (α and β) each with dimmer subunits α1 and α2 plus β1 and β2. There are three major classes of HLA class II, designated HLA-DR, HLA-DP, and HLA-DQ. (Reprinted with permission from Janeway et al[134].) Copyright (2005), Garland Science Books. Permission conveyed through Copyright Clearance Center, Inc.

presenting cells (APC), such as dendritic cells (DC), macrophages, and B lymphocytes. HLA class II molecules are responsible for presenting extracellular antigens that have been internalized, through phagocytosis or antigen receptor mediated internalization, to helper T cells and B cells. Both classes of HLA molecules present foreign and self-

peptides to mature lymphocytes on a regular basis. When a foreign peptide is encountered by an APC, a robust immune response is initiated against all cells containing that foreign peptide. In the case of extracellular pathogens, the pathogen is killed directly by antigen specific lymphocytes. In the case of intracellular pathogens, the host cell is destroyed by the immune response. In the context of transplantation, there are two prominent methods of donor antigen recognition, the direct and indirect pathways (Figure 4.2), which are responsible for graft rejection. In the direct pathway, donor HLA expressed on

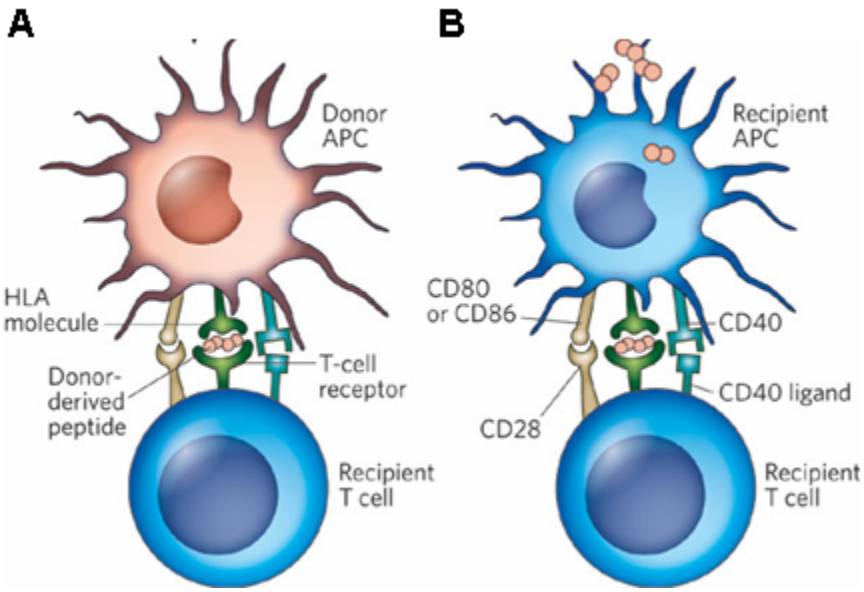

Figure 4.2 Direct and Indirect Pathways of Antigen Recognition. In the context of transplant rejection, there are two pathways of rejection that occur. The direct pathway (A) involves donor APCs being recognized by recipient T cells as being foreign due to the HLA mismatch. The indirect pathway involves recipient APCs presenting donor antigens within the HLA molecule to recipient T cells. (Adapted by permission from Macmillan Publishers Ltd: Nature, Chidgey et al[135].)Copyright (2008)

donor APCs is directly recognized by recipient T cells as being foreign. In the case of the indirect pathway, donor cells slough off antigens that are picked up by recipient APCs and displayed as foreign antigens by the APC HLA to recipient T cells.

In this study, we used islets isolated from deceased donor pancreata to demonstrate induction of HLA class II molecules by inflammatory cytokines (IFN-γ and TNF-α) using real-time PCR analysis, immunofluorescent staining, and flow cytometry. Since 2006, our center has performed 17 islet transplants into nine patients under two investigational drug applications (IND). One of the transplant recipients developed antibodies to a donor HLA class II allele (DR7). Post-transplant serum from this patient showed significant binding to cytokine stimulated islet cells from DR7 positive donors. These results clearly demonstrate that human islets express HLA class II under inflammatory conditions. This could lead to an anti-class II response which, in turn, may play a critical role in rejection of islet allografts.

Materials and Methods

Pancreas Procurement and Islet Isolation

Donor pancreata were procured from deceased multi-organ donors after obtaining consent for research through local Organ Procurement Organizations. Institutional Review Board approval and informed written consent from the patient were obtained prior to study. Organ procurement was performed by the Baylor Transplant Surgeons. All pancreata were procured using a standardized technique to minimize warm ischemia. Pancreata were preserved using the University of Wisconsin/perfluorocarbon (UW/PFC) two-layer method[102] at 4°C for less than eight hours until the islet isolation procedure. Pancreatic islet isolation was performed using the semi-automated method described by Ricordi et al[45]. Islet counts and purity were assessed using dithizone. Viability was assessed using Fluorescein Diacetate/Propidium Iodide (FDA/PI). All islet samples used in this study were greater than 50% pure with a viability of greater than 85%. Islet cell preparations from four HLA-DR7+ donors, and five HLA-DR7- donors were used for experiments (Table 4.2). No statistical difference in purity or viability between islets cells from HLA-DR7+ and HLA-DR7- donors was observed (Table 4.3).

Cytokine Treatment

Equal numbers of isolated islet cells from the same donor were divided into two groups. Group one was placed at 37°C for 48 hours in CMRL supplemented culture medium (Cellgro, Manasas, VA) containing 10% heat inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) in presence of 5% CO_2. Group two was

Table 4.2 HLA Characterization of Donor Islets

BIC ID	HLA-A	HLA-B	HLA-DR	HLA-DQ	Purity	Viability	SI
DR7+							
BIC052R*	1,2	53,63	1,8	5,7	40	97	2.10
BIC053R	1,11	7,57	4,7	8,9	90	95	2.10
BIC056R*	1,2	8,44	17,4	2,7	90	97	2.66
BIC059R	2	13,27	4,7	2,7	70	95	2.30
BIC061R	1,3	7,57	4,7	7,9	50	97.7	3.70
BIC083R	1,3	37,44	1,7	2,5	70	94.6	2.60
DR7-							
BIC057R	1,3	8,62	1,13	5,6	80	90	13.70
BIC062R	2,3	45,58	8,13	6,7	90	99	1.50
BIC063R	1,2	8,44	17,4	2,7	60	80	2.20
BIC064R	24	38,61	8,13	4,6	80	99	18.60
BIC079R	2,31	35,75	4	8	95	99	3.60
BIC081R	2	18,52	4,14	5,8	50	92.3	0.42

* denotes samples used for cytotoxicity assay with HLA-DQ7+ serum

placed under similar conditions in the presence of 100 U/ml (5ηg/ml) interferon gamma (IFNγ) (R&D Systems, Minneapolis, MN), and 1000 U/ml (9.1ηg/ml) tumor necrosis factor alpha (TNFα) (R&D Systems, Minneapolis, MN), as previously described[84,85]. After 48 hours of treatment, islets from

both groups one and two were further divided. Groups 1A and 2A were fixed using 4% paraformaldehyde (PFA) (Sigma

Table 4.3 Quality of Islet Cell Preparations

Quallity	DR7+		DR7-		
Assesment	Mean	SEM	Mean	SEM	p
Purity	68.33	8.33	75.83	7.12	0.51
Viability	96.05	0.54	93.22	3.09	0.39
SI	2.58	0.24	6.67	3.09	0.22

Aldrich, St Louis, MO) at room temperature for 20 minutes immediately upon completion of treatment. Groups 1B and 2B were dispersed into a single cell suspension by adding Accutase™ (Innovative Cell Technologies, San Diego, CA) to the samples and incubating at 37°C for 15 minutes, as previously described[136]. Upon completion of islet dispersion, the single cell suspensions were then fixed as described above. All fixed cells were stored in wash/stain buffer (1% FBS, 0.01% sodium azide (Sigma Aldrich, St Louis, MO) in phosphate buffered saline (PBS; Gibco, Carlsbad, CA)) at 4°C.

Real-Time PCR

A time course study was performed using islets cultured in the control or treatment medium at 3, 6, 18, and 24 hours. If immediate RNA isolation was not possible, RNA content of the single cell suspension was preserved using RNA later or RNA protect (Qiagen, Valencia, CA)

following manufacturer's directions. Total RNA was isolated following the single-step total RNA isolation[137] reagent method using TRI reagent® (Applied Biosystems/Ambion, Austin, TX). One µg total RNA from each sample was used as a template for the formation of cDNA using High Capacity cDNA archive kit (Applied Biosystems, Foster City, CA) following the manufacturer's recommended cycling conditions. Following the formation of the single stranded cDNA, 100 ng of cDNA from each sample was plated in triplicate for each gene of interest. TaqMan® (Applied Biosystems, Foster City, CA) probes/primers for HLA-DRα, CIITA, and HLA-DRβ1 genes were used to assess the relative quantity of each gene transcript compared to a normalizer gene (GAPDH). A relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method. Data was expressed as a percentage of control for each time point. Real-time PCR was performed on a Stratagene Mx3000P real-time PCR machine.

Immunofluorescent Imaging

Aliquots from islet samples in Group 1A and 2A were placed in labeled micro centrifuge tubes. The cells were pelleted by centrifugation, and the supernatant was aspirated off. The pellet was then placed in a cryomold and covered with Tissue Tek ® OCT compound (Sakura Finetek

USA Inc., Torrance, CA) for cryosectioning. Sections were cut at 6 μm increments and placed onto positively charged microscope slides. The sectioned islet samples were allowed to dry and then washed in PBS. The sectioned islet cells were incubated for 20 minutes at room temperature in 10% FBS in PBS. The sections were washed three times in wash/stain buffer. Mouse anti-human HLA-DR7 monoclonal antibody (Abcam, Cambridge, MA) conjugated to fluorescein isothiocyanate (FITC) (Sigma Aldrich, St Louis, MO) was added to the sections in a 1:50 dilution and incubated at room temperature for 30 minutes. After the incubation period the sections were washed three times and then rinsed in permeabilization buffer (PBS supplemented with 0.1% Triton X-100 (Sigma Aldrich, St Louis, MO), 3% BSA (Sigma Aldrich, St Louis, MO), and 0.01% sodium azide). The sections were then incubated in permeabilization buffer at room temperature for 30 minutes. Mouse anti-human insulin, and mouse anti-human glucagon (Abcam, Cambridge, MA) labeled with Alexa fluor-647, and Alexa-fluor-568 respectively (Invitrogen, Carlsbad, CA) were added in a dilution of 1:200 in permeabilization buffer and incubated at room temperature for 30 minutes. After the incubation period, the samples were washed twice in permeabilization buffer, followed by a final wash in wash buffer.

Vectashield (Vector Laboratories, Burlingame, CA) containing DAPI was added to the section, and then covered with a coverslip. Islets were then visualized on an upright fluorescent microscope using MetaMorph software (Molecular Devices, Sunnyvale, CA) for acquisition and analysis of the images. Mouse anti-human IgG1 (R&D Systems, Minneapolis, MN) labeled to the appropriate fluorophore served as isotype control.

Flow Cytometry

Aliquots of approximately 300,000 cells were obtained from samples in Group 1B and 2B. Samples were labeled with a mouse anti HLA class I –AF488 antibody (BioLegend, San Diego, CA), an anti HLA class II –FITC antibody (GeneTex Inc, San Antonio, TX) an anti HLA-DR (eBioscience, San Diego, CA) coupled with goat anti mouse-AF488 (Invitrogen, Carlsbad, CA) antibody, or a mouse anti-HLA-DR7-FITC antibody. Dilutions were as follows: HLA class I, 1:100; HLA class II, 1:10; HLA-DR, 1:500; HLA-DR7, 1:500. In a separate experiment, samples were labeled with a mouse anti-human HLA-DR7-FITC and mouse anti-human insulin-AF647 antibody at a 1:1000 dilution in the same manner as the labeling for imaging was performed. Mouse anti-human IgG1 and mouse anti-human IgG2a labeled with the corresponding

fluorophore served as isotype control. The cells were analyzed on a FACSCaliber (BD Systems, Franklin Lakes, NJ) and the resulting data analyzed using FlowJo 7.2.5 (Tree Star, Ashland, OR).

Examination for Binding of Anti-HLA-DR7 Positive Serum from Islet Transplant Recipient to Cytokine Treated DR7+ Islets

We used post-transplant serum from an islet transplant recipient. This recipient received one dose of 7,504 IEQ/kg allogeneic islets under the Edmonton protocol. The donor HLA alleles were A2, 29; B64, 60; DR7, 13; whereas, the recipient HLA alleles were A2, 3; B7, B8; DR3, 4. The patient did not achieve insulin independence with one infusion and discontinued immunosuppression after 16 months post-transplant. Serum collected post-transplant from the patient and tested by Luminex bead assay showed that the recipient developed anti-DR7 specific antibodies (mismatched donor class II antigen). We used serum from this patient (monospecific) to test for binding to HLR-DR7 positive islets treated with cytokines, whereas islets from DR7 negative donors served as control. Aliquots of approximately 300,000 cells were obtained from islet samples in group 1B and 2B as described above. Sera from normal or patient were incubated with islet cells from HLA-DR7 negative donors to remove any non-specific antibodies.

Islet cells from groups 1B and 2B were incubated with 100 μl of either pooled sera from high PRA patients (positive control), or patient or normal individual diluted 1:2 in PBS for 30 minutes at 37°C. Islets were washed in wash/stain buffer and labeled with goat anti-human IgG-AF488 (Invitrogen, Carlsbad, CA) and goat anti-human IgM-PE (Sigma Aldrich, St Louis, MO) at a 1:100 dilution. The cells were analyzed on FACS Calibur using FlowJo 7.2.5 software.

Complement Mediated Cytotoxicity Assay

Upon completion of the treatment period, an aliquot of islet cells was taken from each condition. The islets were washed, and re-suspended in Accutase for dispersion into single cell suspension by incubation at 37°C for 20 minutes. The single cell suspension was then washed in sterile PBS, and then 1000 single cells were aliquoted into three microfuge tubes for each condition. For each condition, pooled high PRA serum (kindly provided by the Transplant Immunology Laboratory, Baylor University Medical Center) was added to one labeled tube as a positive control, patient serum positive for anti-HLA-DR7 antibodies was added to one labeled tube, and normal patient serum was added to the final labeled tube as a negative control.

Prior to serum treatment, viability was assessed for each condition using Trypan Blue (Sigma, St Louis, MO) exclusion on an aliquot of cells. The six total tubes were incubated at 37°C for 30 minutes. Upon serum treatment, the cells were washed in sterile PBS to remove excess unbound serum antibodies. Next, each tube was aliquoted into three wells on a 96 well plate, for a total of nine wells per initial cytokine treatment condition. To each serum condition, rabbit serum as a source of complement was added at 10% concentration. The 96 well plate was then incubated at 37°C for two hours for complement activation and cytotoxicity. Upon completion of the complement treatment period, each well was assessed for cytotoxicity as measured by Trypan Blue Exclusion. The post-complement viability was corrected against the viability prior to serum treatment. Each well was counted twice and the average viability was calculated.

Statistical Methods

Grubbs test for significant outlier detection was used to determine possible outliers. Outliers were withheld from further statistical analysis. Students one-tailed T-test was used to determine statistical significance.

Results were considered to be statistically significant when the p-value was <0.05.

Results

IFNγ and TNFα Treatment of Isolated Human Pancreatic Islets Results in Up-Regulation of CIITA, HLA-DRα, and HLA-DRβ1 Transcripts

To determine the effect of inflammatory conditions on the expression of HLA class II molecules, we evaluated the result of IFNγ and TNFα treatment on the expression of MHC class II trans-activator (*CIITA*), and human leukocyte antigen class II transcripts (*DRα* and *DRβ1*) in isolated pancreatic islet cells. Using real-time PCR analysis on islet cells cultured in the presence of IFNγ (100U/ml) and TNFα (1000U/ml) for 3, 6, 18 and 24 hours, we observed a significant increase ($p<0.05$) in the transcription of *CIITA*, *HLA-DRα*, and *HLA-DRβ1* mRNA relative to untreated control islet samples (Figure 4.3A, 4.3B, and 4.3C). When normalized to *GAPDH* control, *CIITA* gene expression increased to 2.96 fold at 3 hours and subsequently increased 5.07 fold at 6 h, 9.88 fold at 18 h and 9.38 fold at 24 hours (Figure 4.3A). For the same time intervals, *HLA DRα* gene expression increased to 7.07, 6.62, 11.49, and 18.95 fold (Figure 4.3B) and *HLA-DRβ1* gene expression

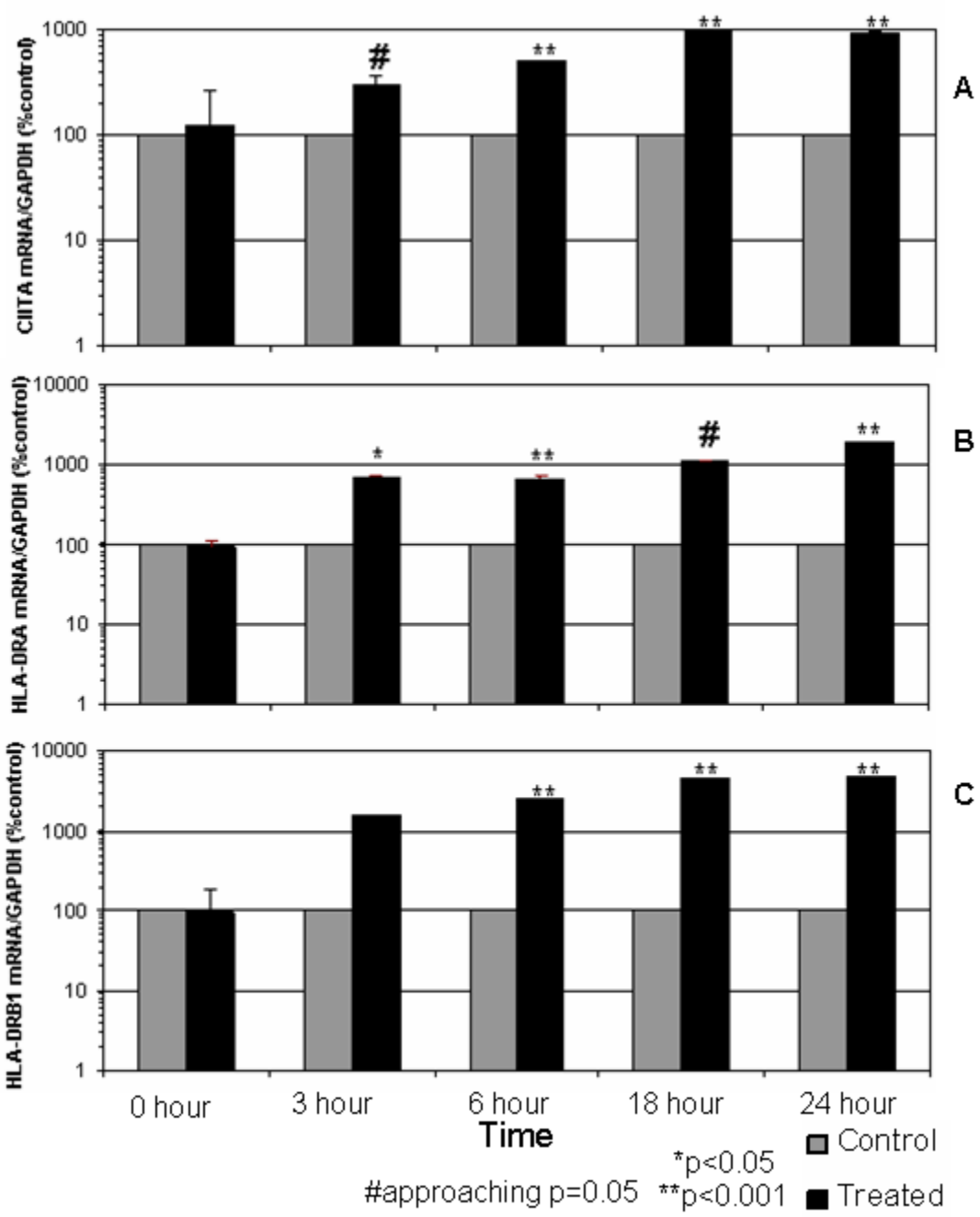

Figure 4.3 Induction of class II human leukocyte antigen genes in isolated human islet cells by treatment with IFNγ and TNFα. Real-time PCR analysis of gene transcripts normalized to *GAPDH* shows up-regulation of *CIITA* (A) at 3, 6, 18, 24 hours (296%, 507%, 988%, 938%; p=0.6, 1.3x10-5, 0.0008, 0.0002) respectively. *HLA-DRA* (B) gene expression at 3, 6, 18, 24 hours (707%, 662%, 1149%, 1895%; p= 0.004, 0.0006, 0.051, 1.05x10-6) respectively. *HLA-DRB1* (C) gene expression at 3, 6, 18, 24 hours (1558%, 2580%, 4556%, 4650%; p= 0.109, 2.6x10-11, 0.0004, 1.23x10-7) respectively.

increased to 15.58, 25.8, 45.56, and 46.5 fold (Figure 4.3C). The up-regulation of these gene transcripts is of key importance to the expression of HLA class II antigens on the surface of isolated islet cells.

HLA Class II Expression on Human Islets Treated With Cytokines

To substantiate the real-time PCR data showing the increase in HLA class II genes in human islets, we evaluated the result of IFNγ and TNFα treatment on the surface expression of HLA class II molecules. Using multi-color immunofluorescent staining, we were able to visualize HLA class II molecules on the surface of cryosectioned isolated human islet cells. Quadruple fluorescence imaging, using monoclonal antibodies specific for insulin, glucagon, and HLA-DR7, along with DAPI for nuclear staining, revealed that HLA class II molecules are present on pancreatic beta cells and alpha cells within the islets (Figure 4.4 –I, J, K). Control islets did not express detectable amounts of HLA- DR7 on their surface (Figure 4.4E). Islet cells from HLA-DR7 negative donors treated with the cytokine medium did not demonstrate binding of the FITC conjugated HLA-DR7 mAb (not shown).

To corroborate data from the imaging analysis, we analyzed the surface expression of HLA class II by flow

cytometry using dispersed islet cells. Staining for change in HLA class I expression, used as a control to determine

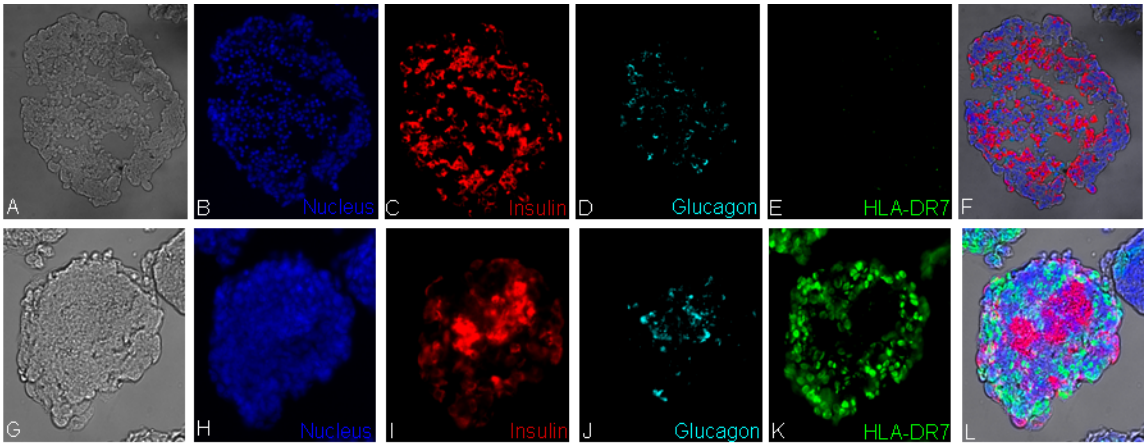

Figure 4.4 Islet cells express HLA-DR7 on their surface following treatment with TNFα and IFNγ. Islet cells seen at 200 magnification in bright field (A, G), labeled for nuclear staining (B, H), insulin (C, I), glucagon (D, J), HLA-DR7 (E, K), and overlay (F, L). Control islets (A-F) show no staining for HLA-DR7, while the cytokine treatment of islets (G-L) shows a remarkable increase in expression of HLA-DR7. HLA-DR7 expression appears to not be expressed in strongly positive alpha and beta cells, while weakly positive alpha and beta cells express HLA-DR7.

the effect of cytokine treatment, showed dramatic increase (Figure 4.5A). Similarly, staining with a HLA class II antibody and a more specific HLA-DR antibody also showed remarkable increase in class II molecules (Fig. 4.5B). Based on the shape of the peak, it is evident that most of the cell types contained in human islets expressed HLA class II molecules when treated with TNFα and IFNγ. We also tested islets isolated from HLA DR7 positive donors, to measure expression of HLA-DR7 on the surface of cytokine

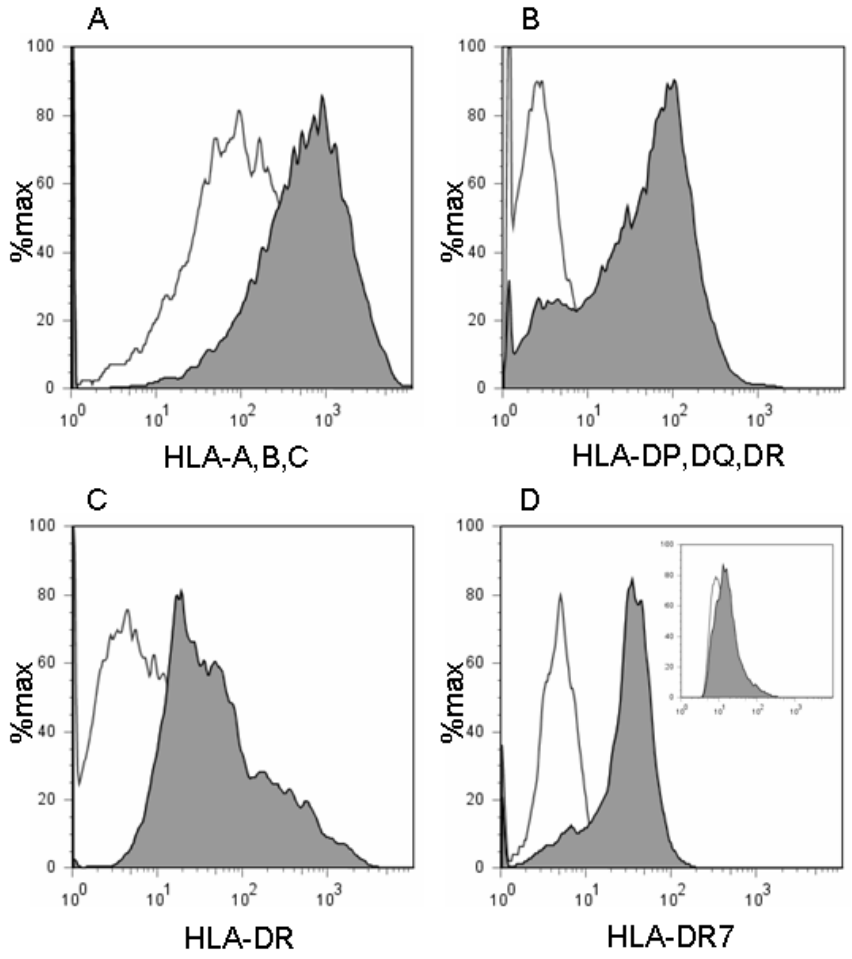

Figure 4.5 Up-regulation of HLA-DR Expressed on Islet Cells. FACS analysis showed an increase in HLA expression in cytokine treated cells (solid grey) when compared control (black line). HLA class one was up-regulated by cytokine treatment (A). HLA class two was up-regulated by cytokine treatment (B). HLA DR positivity was observed in all islet constituent cells (C). Further staining for subtype HLA-DR7 shows an increase in cytokine treated cells (D). Gating for insulin positive cells (D inset) showed that beta cells express also HLA-DR7 on their surface.

treated islets. As shown in Fig. 4.5C, we could observe significant expression of DR-7 on cytokine treated islets. Selecting the beta cells by gating on insulin positive staining from the islet cell constituents revealed that under inflammatory conditions the human islet beta cells

have induced expression of HLA class II molecules (Figure 4.5D). Collectively, these results showed that following cytokine treatment, expression of HLA class II molecules is significantly up-regulated on islets including beta cells.

Islet Transplant Recipient Serum Positive for Anti-HLA-DR7 Binds to Cytokine Treated DR7+ Islets

Control and cytokine treated islet cells from a HLA-DR7+ donor were dispersed into single cell suspension, and then incubated with serum from the patient, as well as from normal pooled serum. Prior to incubation, both normal and patient serum were cross absorbed with HLA-DR7 negative islets to remove non-specific binding because the patient's serum may have autoreactive antibodies to islets. Both human IgG and IgM binding to HLA-DR7 positive cytokine treated islet cells was observed, whereas the untreated control islet cells showed no binding (Figure 4.6). Conversely, performing the same treatment on islet cells from an HLA-DR7 negative donor demonstrated no detectable antibody binding in the patient's serum.

Donor Specific Antibodies Against HLA-DR7 Induce Complement Mediated Cytotoxicity in Islet Cells Treated with IFN-γ and TNF-α

In an attempt to demonstrate that donor HLA specific antibodies developed in transplant recipients due to

inflammation can harm the transplanted islet graft, islet cells that were exposed to inflammatory conditions were assessed for complement mediated cytotoxicity. Viable islet cells from control and cytokine treated groups were

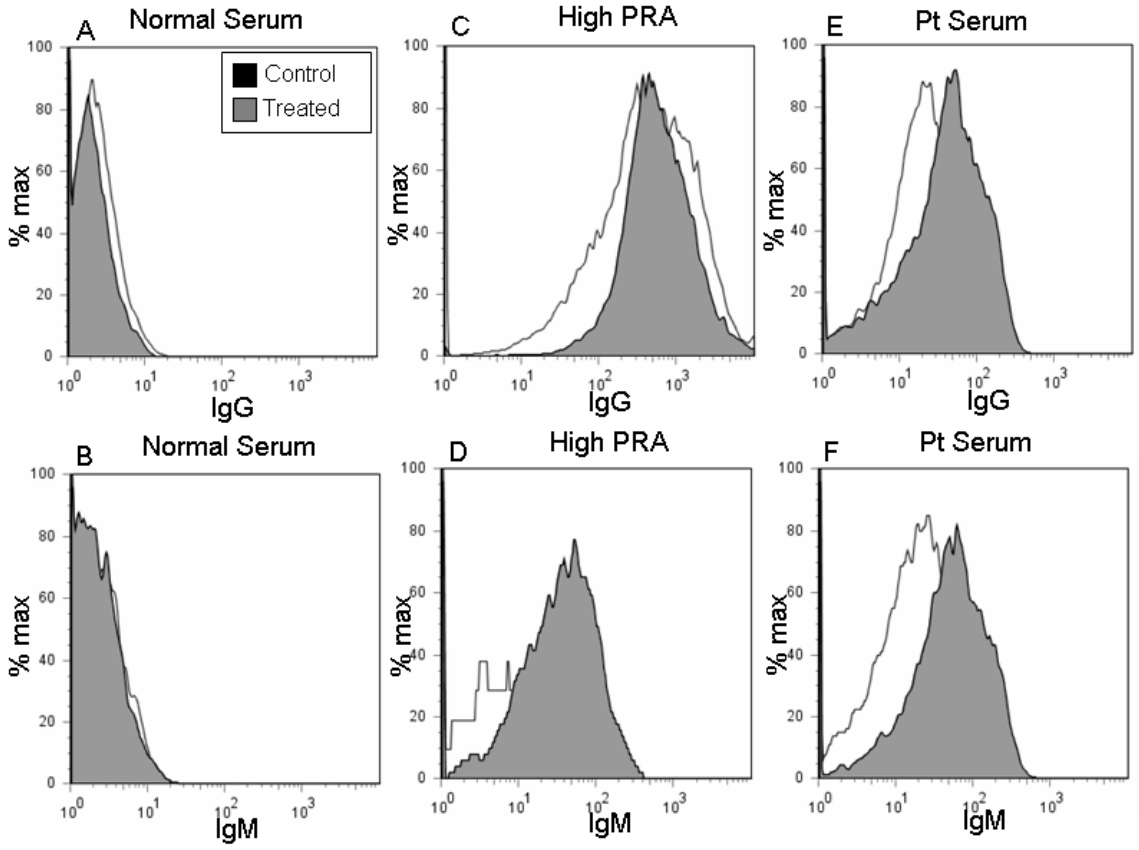

Figure 4.6 Islet transplant recipient anti-HLA-DR7 binds to HLA-DR7+ islet cells. Serum containing monospecific antibodies to HLA-DR7 was tested along with pooled PRA serum (positive control) and pooled normal serum (negative control) for IgG and IgM binding to control and cytokine treated islets by flow cytometry. Pooled normal serum showed negative binding for IgG and IgM (A,B) with negligible difference between conditions. Pooled PRA serum showed positive binding for IgG and IgM (C,D) with slight increase for cytokine treated islets. Patient serum clearly showed increased IgG and IgM binding (E, F) for cytokine treated islets where as the control islets showed negligible binding. Prior to testing, patient serum was pre-absorbed with DR7 negative islets to remove auto as well as non-specific antibodies. Results shown are representative of three independent experiments.

pre-incubated with either patient serum or high PRA serum to allow for antibody engagement of the induced HLA molecules. Next, a complement source was added to the islet cells to induce complement mediated cytotoxicity. Cytokine treated islet cells pre-incubated with PRA serum containing antibodies to all HLA exhibited a higher degree

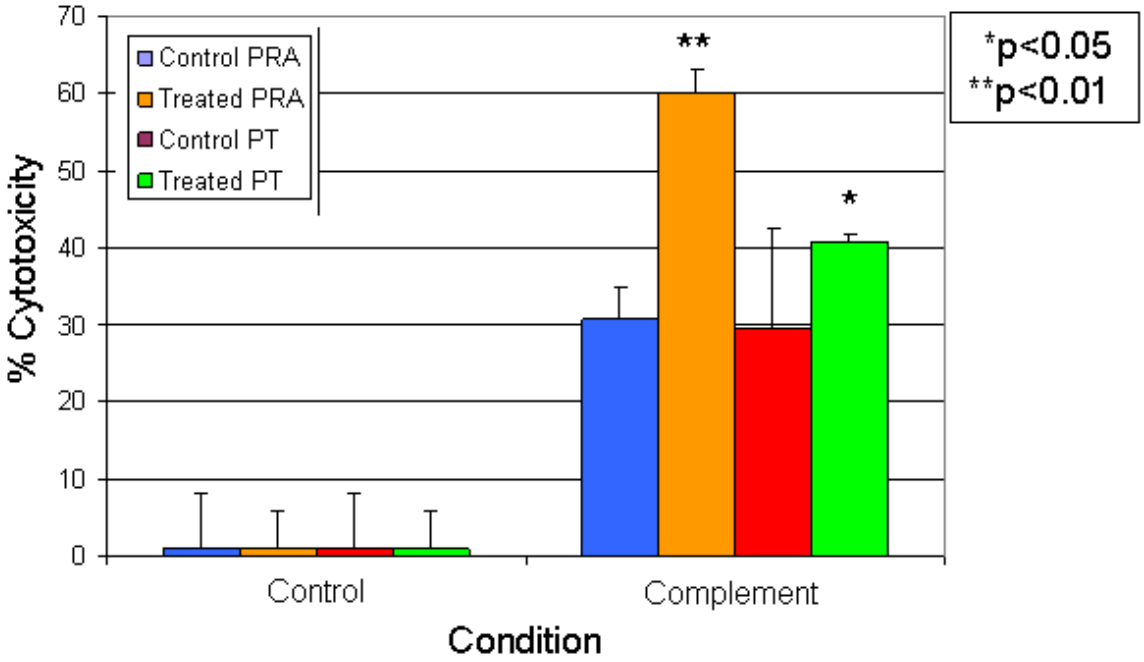

Figure 4.7 Treatment with IFN-γ and TNF-α Leads to Increased Complement Mediated Cytotoxicity. Cytokine treatment of islet cells with IFN-γ and TNF-α induces the expression of HLA class II, and up-regulates expression of HLA class I. The induced expression of HLA-class II is able to cause complement mediated cytotoxicity in the presence of donor specific antibodies and complement, 41% cytotoxicity after complement activation versus 0% cytotoxicity prior to complement activation (p=0.0394). Because the cytokine treatment up-regulates HLA class I expression, High PRA serum also causes complement mediated cytotoxicity, 60% cytotoxicity after complement activation versus 0% cytotoxicity prior to complement activation (p=0.0045).

of cytotoxicity (60.19 ± 2.91, 30.72 ± 3.92)compared with control islet cells respectively. Likewise, cytokine

treated islet cells pre-incubated with patient serum containing donor HLA specific antibodies exhibit a higher degree of cytotoxicity (40.78 ± 0.97, 29.41 ± 13.07) when compared to control islet cells respectively under the same conditions (Figure 4.7). These results were significant ($p<0.5$) when compared to the viability at the beginning of the assay.

Discussion

We demonstrate here that induced expression of HLA class II molecules, brought on by the combination of inflammatory cytokines IFNγ and TNFα, at previously reported concentrations of 100 and 1000 U/ml[85] respectively, could be a factor in the sensitization of islet cell transplant recipients. Real-time PCR analysis demonstrated that low concentrations of these cytokines are sufficient to induce up-regulation in the genes necessary for the translation of HLA class II. Moreover, immunofluorescence and flow cytometric analyses confirmed that the up-regulation of gene transcription translates into increased cell surface expression of HLA class II. This is in agreement with the body of early literature showing induced HLA class II expression in cultured islet monolayers[84]. More importantly, anti-donor HLA class II antibody from an

islet transplant patient serum bound to cytokine treated HLA matched islets, further substantiating our hypothesis that induction of HLA class II expression on islet cell surface leads to a donor specific alloimmune response.

Our data also suggests that cytokine treatment of islets is capable of causing the expression of HLA class II molecules on the surface of beta and alpha cells. During the onset of diabetes, beta cells have been shown to have expression of HLA DR antigens on their surface[138] leading to insulitis[139] and eventual immune destruction of the pancreatic beta cells. It is plausible that the induction of donor specific HLA class II molecules, brought on by inflammatory cytokines, could lead to phenomena similar to the onset of diabetes mellitus. It is well established that concentrations of 1000 U/ml IFNγ induces expression of HLA class II molecules in thyroid follicular cells, as well as pancreatic exocrine and ductal cells[83,84,140]. However, pancreatic islet cells are known to respond differently to IFNγ by inducing HLA class I molecules[141]. IFNγ coupled with TNFα at equal concentrations have been demonstrated to induce HLA class II expression in monolayer cultured islet cells[84]. However, this concentration is associated with cytotoxicity to the islets[133]. We demonstrated here that this combination of IFNγ plus TNFα, at lower

concentrations, is also able to induce HLA class II expression in freshly isolated human pancreatic islet cells, while avoiding cytotoxicity. Following intraportal infusion of allogeneic islets, the transplanted islet mass is subjected to inflammatory cytokines[142,143]. This environment could potentially lead to the induced expression of HLA class II molecules on the transplanted islet cell mass, culminating in the sensitization of the recipient to the islet graft.

Our data demonstrates a significant increase in complement mediated cytotoxicity in cytokine treated islet cells compared to control. As our data indicates, HLA class I and HLA class II are both up-regulated in surface expression on pancreatic islet cells. Compared with the control islet cells, the cytokine treated islet cells treated with both high PRA serum and patient serum demonstrated increased cytotoxicity. Taken in the context of a sensitized transplant patient, the increased expression of HLA molecules, class I and/or class II, brought on by inflammatory conditions could lead to the development of donor specific antibodies. The development of donor HLA specific antibodies could initiate complement mediated cytotoxicity leading to graft destruction.

The role of HLA antibodies in islet graft survival is not clear. Islet transplants performed at many centers immediately following the reporting of Edmonton protocol did not require the recipients to be screened for the presence of class II panel reactive antibodies. However, several reports using sensitive detection methodologies have now documented the presence of HLA antibodies in islet transplant recipients during both the pre- and post-transplant period[77-81,144]. Two recent reports[79,80] have shown that the presence of anti-HLA antibodies may adversely influence the outcome of islet transplants, whereas another report[144] suggested further evaluation before drawing conclusions on their role. Our data suggests that antibodies developed against donor HLA class I and II are capable of activating the complement cascade, leading to complement mediated cytotoxicity. This could have a devastating result due to the lack of B cell targeted immune suppression that plays an important role in the development of donor specific antibodies, and the activation of the complement cascade.

Currently, immune suppression is targeted mainly against the T cell immune response, with very little thought towards specifically suppressing the B cell response with targeted antibodies. Recently, a study

looking at the ability of B lymphocyte targeted immunosuppression using Rituximab to induce tolerance reported some significant findings[145] in a non-human primate model. This study found that Rituximab in combination with Anti-thymocyte globulin (ATG) and limited maintenance therapy with Rapamycin was able to induce long-term graft survival when maintenance therapy was ceased. They also concluded that those recipients that had long term graft survival maintained a large population of immature B lymphocytes upon B cell repopulation. Targeting B lymphocytes could prove useful in the setting of HLA sensitization prevention.

In the transplant setting, acute graft rejection is primarily mediated through direct T cell rejection, while chronic graft rejection is mediated through indirect T cell recognition[146]. In the direct response, the donor HLA molecules are directly recognized by host effector T cells as foreign antigens, which leads to the CD8 T cell mediated rejection of the transplanted graft. Because our data shows that HLA expression on islet cells is up-regulated by inflammation, as demonstrated by treatment with TNFα and IFNγ, the transplanted islet graft is more susceptible to CD8 direct recognition of donor class I and to CD4 direct recognition of donor class II pathways of rejection[147,148].

An alternative mechanism could be the indirect pathway[147,148] of antigen recognition, in which donor antigens are shed off of the islet graft to be picked up and presented by HLA class II on antigen presenting cells (APC) to effector T cells. This could explain the delayed graft rejection seen in many islet transplant recipients, as donor antigens can remain in APCs for extended periods of time, until a lapse in immune suppression makes available naïve T cells ready for antigen activation.

In conclusion, our data suggests that the inflammatory environment encountered by the transplanted islet mass shortly after transplantation leads to the induced expression of HLA class II molecules on the surface of the islet cell. The expression is not localized solely to the beta cell, but is also expressed on the alpha cell. Donor specific HLA class II antibodies developed in an islet transplant recipient showed binding to cytokine treated islets, further supporting our hypothesis. Further studies will focus on the consequence of binding of HLA class II antibodies to cytokine stimulated islets. Previous studies have shown that HLA-DR ligation mediates cell death of antigen-presenting cells (APC), including mature B cells, macrophages, and dendritic cells[149,150]. Similar mechanisms for beta cells of islets could explain the high risk of

islet transplant failure in patients with HLA sensitization.

CHAPTER FIVE

Summary and Conclusions

Summary

Prolonged Cold Ischemia has a Detrimental Effect on the Outcome of Islet Cell Isolation

Our data demonstrates that for optimal pancreatic islet cell isolation, the cold ischemic organ preservation should be kept to less than four hours. This is in contrast to previous reports[88] which demonstrate that cold ischemic preservation of the human pancreas should be less than eight hours when preserved by the single layer (UW) method, or less than twelve hours when preserved by the two layer method. Our data demonstrates a significant decrease in islet cell yield in both the pre-purification enumeration and the post-purification enumeration. This is important because one of the main release criteria for pancreatic islet cell transplantation is that the dose of islet cell mass transplanted should be equal to or greater than 5,000 IEQ/kg of recipient's body weight. By reducing the cold ischemic preservation, we can statistically increase the quantity of islet cells recovered during the

isolation process which, in turn, increases the transplant rate.

It is also plausible that increasing the islet yield from the isolation process could lead to less secondary transplants to attain adequate islet cell mass for the achievement of insulin independence. This is of importance because a major obstacle in the field of islet cell transplantation is the shortage of organs. By reducing the number of pancreata needed for each individual patient to achieve insulin independence, we can make more islet cell transplantations available to other diabetic patients and thus increase the efficiency in the use of precious donor organs. Reducing the number of islet transplants will also benefit the patient in other respects. Because of the shortage of pancreata needed for islet cell transplantation, islet transplant centers do not match the donor and recipient for histocompatibility. Reducing the number of islet cell transplants would have two benefits. First, in the event that islet cell transplant centers continue to transplant histocompatibility mismatched islet cells, the recipient would be exposed to fewer mismatches, greatly improving the recipient's chances of a secondary kidney transplant if sensitization occurs. Second, if insulin independence is consistently attained with a single

islet cell transplantation, islet cell transplant centers may begin to perform matched islet cell transplants.

The Role of Complement in the Destruction of Islet Grafts Post-Transplant

We demonstrated that autoantibodies present in type 1 diabetic patient serum prior to transplant are able to recognize autoantigens present on the surface of donor pancreatic islet cells and engage complement fixation on the surface of transplanted islet cell grafts. The fixation of complement on the surface of the islet cell initiates a response within the islet that leads to the attraction of the immune system and the removal of inhibitors of cytokine signaling and oxidative stress. This is of key importance because the loss of islet grafts during the initial post-transplant setting is not completely understood. Certainly, a large proportion of the transplanted islet graft would become trapped within clots initiated by the coagulation cascade and IBMIR. However, those islets that survive coagulation and lymphocyte infiltration are still vulnerable to the remnants of the immune system.

The Expression of HLA Class II Molecules on the Surface of Isolated Islet Cells and their Role in Sensitization and Rejection

Our data clearly demonstrates that pancreatic islet cells, in particular pancreatic beta cells, are capable of inducing HLA class II expression on the cell surface. This is in agreement with previous reports based upon the mouse model[85] beta cell line MIN6, as well as reports using cultured islet cells from chronic pancreatitis[84]. To our knowledge this is the first report of HLA class II being expressed on the surface of healthy islet cells.

This finding is of significance because islet cell grafts are susceptible to both autoimmune recognition and alloimmune recognition within a type 1 diabetic recipient[151]. The development of methods to overcome the autoimmune, as well as the alloimmune response, is of great importance to the forward progress of islet cell transplantation.

We observed that inflammation was able to induce the expression of HLA class II on the surface of islet cells. This aberrant expression could lead to both CD4 direct recognition of donor HLA class II and to CD4 indirect recognition of donor peptides in the context of recipient HLA class II antigen presentation on the surface of recipient APCs[152]. Recent work has exploited a mechanism

for inducing tolerance to islet grafts in a mouse model by manipulating the CD4 indirect pathway[152]. The authors of this study observed that indirect immunity was not effective in eliminating donor T cell rejection, which led to a state T cell chimerism. The chimerism led to the tolerance of a second graft.

Similar work has also been done using a xenograft model of islet transplantation[153]. Using two CTLA4-Igs, specific for porcine co-stimulatory molecule B7, and murine B7, they were able to inhibit both the direct and indirect responses respectively. By eliminating both the direct and indirect pathways of T cell mediated rejection, the authors were able to prevent islet cell graft rejection in the absence of immunosuppression.

Indeed, much attention has been paid to the cell-mediated immune response to allogeneic islet cell grafts. However, the nature of the indirect pathway involves APCs presenting donor antigen to recipient CD4 T cells. It is plausible that APCs could activate B cells leading to the development of memory B cells and plasma cells. Because the immunosuppressive regimen of islet transplantation is directed against cell mediated immunity, rather than humoral immunity, the activated B cells are free to proliferate and differentiate into plasma cells specific to

the donor antigen, and memory B cells. Antibodies directed against donor antigens are known to be both complement-fixing and non-complement-fixing[154]. We demonstrated in our data that donor specific antibodies directed against HLA class II are complement fixing antibodies. Because the antibodies developed in the recipient are complement-fixing, the antibodies are able to cause the rejection of the islet graft by complement mediated cytotoxicity.

Conclusions

In conclusion, the findings presented herein have profound impact on the field of islet cell transplantation, both in process of the isolation and in the management of the patient in three areas. First, our data clearly demonstrates the need to limit the preservation of the pancreas to less than four hours to significantly improve the outcome of the pancreatic islet cell isolation process. The reduction in the preservation results in the possibility for a reduction in the number of islet cell transplants to achieve insulin independence. This also will add the benefit of reducing the number of HLA mismatches to which the patient would be exposed.

Second, the discovery of the gene expression response in pancreatic islet cells toward complement and

autoantibody binding reveals possible interventional therapies. Our laboratory has already begun using a drug, known as Anakinra, designed to block the signaling of IL-1β. The idea for this interventional therapy arose from the discovery that pancreatic islet cells are down-regulating the transcription of the IL-1 receptor antagonist (*IL-1RN*) in response to autoantibody-mediated complement fixation. By reducing the inflammatory cytokine signaling, which leads to apoptosis, the transplanted islet cell graft has an increased chance of engraftment and survival.

Third, finding that pancreatic islet cells express HLA class II upon inflammation can lead to the targeting of inflammatory cytokines during the transplant period. Perhaps reducing the amount of inflammation experienced by the islet cell graft could lead to prolonged survival of the graft by reducing the exposure of donor HLA to circulating recipient T cells and APCs.

Collectively, these results reveal potential avenues for the improvement of islet cell transplantation. My results reveal areas for improvement in the procurement of the donor pancreas, and for the treatment of the patient receiving the islet cell graft. The field of islet cell transplantation is in great need of improvement if it is to

become the standard therapy for the treatment of type 1 diabetes mellitus. These results point to potential interventions that could lead to the results needed for the improved success of islet cell transplantation.

APPENDIX

APPENDIX

Author Permissions

JOURNAL OF CLINICAL INVESTIGATION
Order detail ID: 25768690

ISBN/ISSN: 00219738
Publication Year: 2005
Publisher: AMERICAN SOCIETY FOR CLINICAL INVESTIGATION
Rightsholder: American Society for Clinical Investigation
Author/Editor: M. Trucco

Permission Status: **Granted**

Permission type: Republish into a book, journal, newsletter…

Requested use: Dissertation
Republication title: ANALYSIS OF INFLAMMATORY CHANGES IN HUMAN PANCREATIC ISLET CELLS
Republishing organization: UMI
Organization status: Not for profit
Republication date: 05/16/2009
Circulation/Distribution: 3
Type of content: Illustration
Description of requested content: structure of pancreatic islet cell
Page range(s): 5-12
Requested content's publication date: 01/01/2005

Your reference: DISSERTATION CHAPTER 1

Confirmation Number: 2024046

Special Rightsholder Terms & Conditions
The following terms & conditions apply to the specific publication under which they are listed

JOURNAL OF CLINICAL INVESTIGATION
Order Detail ID: 25768690

Must state work is being republished. The American Society for Clinical Investigation will allow adaptations to be made to the original work pending review. If you wish to modify the original work in any way, please fax a copy of both the original work and the modified version along with your CCC order number to 978-646-8633. Republication of this JCI content is permitted in print format and in the corresponding online version. All JCI content must be identified as such in print and in all online versions.

Copyright Clearance Center Terms and Conditions
The following terms & conditions apply to your entire order.

For uses traditionally reported under Copyright Clearance Center's (CCC) pay-per-use service to photocopy for general business use, library reserves, ILL/document delivery, etc., (formerly TRS), the applicable terms and conditions follow immediately below in Section A. For republication or

event that the material for which an electronic reproduction license is sought includes third party materials (such as photographs, illustrations, graphs and similar materials) which are identified as included in a Work by permission, such third party materials may not be reproduced except in the context of the Work. Unless otherwise provided in the Order Confirmation, any grant of rights to User (i) is "one-time", (ii) is non-exclusive and non-transferable, and (iii) is limited to use completed within 30 days for any use on the Internet, 60 days for any use on an intranet or extranet and one year for any other use, all as measured from the "republication date" as identified in the Order Confirmation, if any, and otherwise from the date of the Order Confirmation. Upon completion of the licensed use, or at the end of the period identified in the previous sentence (if earlier), User shall immediately cease any new use of the Work(s) and shall destroy or (particularly in the case of electronic republications) render inaccessible (such as by removing or severing links or other locators) any further copies of the Work (except for copies printed on paper in accordance with this license and still in User's stock at the end of such period). All rights not expressly granted are reserved; any license granted is further limited as set forth in any restrictions included in the Order Confirmation and/or in these terms and conditions.

3. Use of proper copyright notice for a Work is required as a condition of any license granted hereunder. Unless otherwise provided in the Order Confirmation, a proper copyright notice will read substantially as follows: "Used [or, as appropriate, "Reproduced" or "Republished"] with permission of [Rightsholder's name], from [Work's title, author, volume, edition number and year of copyright]; permission conveyed through Copyright Clearance Center, Inc." Such notice must be placed immediately adjacent to the Work as used (for example, as part of a by-line or footnote but not as a separate electronic link), in a reasonably legible font size, unless, in the case of a republication license, the new work containing the republished Work contains a substantial number of credits or copyright notices and they are all printed in a single, easily located place. Failure to include the required notice results in loss to the Rightsholder and CCC, and User hereby agrees to pay liquidated damages for each such failure equal to twice the use fee specified in the Order Confirmation, in addition to the use fee itself and any other fees and charges specified.

4. User may not make or permit any alterations to the Work, unless expressly set forth in the Order Confirmation (after request by User and approval by Rightsholder). No Work may be used in any way that is defamatory, violates the rights of third parties (including such third parties' rights of copyright, privacy, publicity, or other tangible or intangible property), or is otherwise illegal, sexually explicit or obscene. In addition, User may not conjoin a Work with any other material that may result in damage to the reputation of the Rightsholder.

5. User agrees to inform CCC if it becomes aware of any infringement of any rights in a Work and to cooperate with any reasonable request of CCC or the Rightsholder in connection therewith.

6. User hereby indemnifies and agrees to defend the Rightsholder and CCC, and their respective employees and directors, against all claims, liability, damages, costs and expenses, including legal fees and expenses, arising out of any use of a Work beyond the scope of the rights granted herein, or any use of a Work which has been altered in any way by User, including claims of defamation or infringement of rights of copyright, publicity, privacy or other tangible or intangible property.

7. LIMITATION OF LIABILITY. UNDER NO CIRCUMSTANCES WILL CCC OR THE RIGHTSHOLDER BE LIABLE FOR ANY DIRECT, INDIRECT, CONSEQUENTIAL OR INCIDENTAL DAMAGES (INCLUDING WITHOUT LIMITATION DAMAGES FOR LOSS OF BUSINESS PROFITS OR INFORMATION, OR FOR BUSINESS INTERRUPTION) ARISING OUT OF THE USE OR INABILITY TO USE A WORK, EVEN IF ONE OF THEM HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. In any event, the total liability of the Rightsholder and CCC (including their respective employees and directors) shall not exceed the total amount actually paid by User for this license. User assumes full liability for the actions and omissions of its principals, employees, agents, affiliates, successors and assigns.

8. WARRANTIES. THE WORK(S) AND RIGHT(S) ARE PROVIDED "AS IS". CCC HAS THE RIGHT TO GRANT TO USER THE RIGHTS GRANTED IN THE ORDER CONFIRMATION DOCUMENT. CCC AND THE RIGHTSHOLDER DISCLAIM ALL OTHER WARRANTIES RELATING TO THE WORK(S) AND RIGHT(S), EITHER EXPRESS OR IMPLIED, INCLUDING WITHOUT LIMITATION IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. ADDITIONAL RIGHTS MAY BE REQUIRED TO USE PORTIONS OF THE WORK (AS OPPOSED TO THE ENTIRE WORK) IN A MANNER CONTEMPLATED BY USER; USER UNDERSTANDS AND AGREES THAT NEITHER CCC NOR THE RIGHTSHOLDER MAY HAVE SUCH ADDITIONAL RIGHTS TO GRANT.

9. The licensing transaction described in the Order Confirmation is personal to User. Therefore, User

IMMUNOBIOLOGY 6 - CHURCHILL LIVINGSTON EDITION
Order detail ID: 25768692

ISBN/ISSN: 9780443073106
Publication Year: 2005
Publisher: GARLAND SCIENCE
Rightsholder: Garland Science - Books
Author/Editor: Janeway et al

Permission Status: **Granted**

Permission type: Republish into a book, journal, newsletter…

Requested use: Dissertation
Republication title: ANALYSIS OF INFLAMMATORY CHANGES IN HUMAN PANCREATIC ISLET CELLS
Republishing organization: UMI
Organization status: Not for profit
Republication date: 05/16/2009
Circulation/Distribution: 3
Type of content: Illustration
Description of requested content: structure of MHC
Page range(s): 122-123
Requested content's publication date: 01/01/2005

Your reference: DISSERTATION CHAPTER 4

Permission is granted for your use of the figure as described in your message below. Please cite the full journal references and "Copyright (copyright year) National Academy of Sciences, U.S.A."

Cabrera O, Berman DM, Kenyon NS, Ricordi C, Berggren PO, Caicedo A. The Unique Cytoarchitecture of Human Pancreatic Islets has Implications for Islet Cell Function. Proc. Natl. Acad. Sci. USA. 2006; 103(7):2334-2339.

Siyuan Xie for
Diane Sullenberger
Executive Editor
PNAS

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS
Feb 27, 2009

This is a License Agreement between Andrew M Jackson ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2137110154511
License date	Feb 27, 2009
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature
Licensed content title	Tolerance strategies for stem-cell-based therapies
Licensed content author	Ann P. Chidgey, Daniel Layton, Alan Trounson, Richard L. Boyd
Volume number	
Issue number	
Pages	
Year of publication	2008
Portion used	Figures / tables
Requestor type	Student
Type of Use	Thesis / Dissertation
High-res requested	No
Billing Type	Invoice
Company	Andrew M Jackson
Billing Address	4210 Wild Plum Dr Carrollton, TX 75010 United States
Customer reference info	
Total	0.00 USD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this

material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.
5. The credit line should read:

   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

   For AOP papers, the credit line should read:

   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should be credited as follows:

   Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit

http://www.macmillanmedicalcommunications.com for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

v1.1

WOLTERS KLUWER HEALTH LICENSE
TERMS AND CONDITIONS

Feb 27, 2009

This is a License Agreement between Andrew M Jackson ("You") and Wolters Kluwer Health ("Wolters Kluwer Health") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Wolters Kluwer Health, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2137260851041
License date	Feb 27, 2009
Licensed content publisher	Wolters Kluwer Health
Licensed content publication	Transplantation
Licensed content title	Evidence for Induced Expression of HLA Class II on Human Islets: Possible Mechanism for HLA Sensitization in Transplant Recipients.
Licensed content author	Jackson, Andrew
Licensed content date	Jan 1, 2009
Volume Number	87
Issue Number	4
Type of Use	Dissertation/Thesis
Requestor type	Individual
Order reference number	chapter 4 permission
Title of your thesis / dissertation	Analysis of Inflammatory responses in human pancreatic islet cells
Expected completion date	May 2009
Estimated size(pages)	110
Billing Type	Invoice
Billing Address	4210 Wild Plum Dr

Carrollton, TX 75010

United States

Customer reference info

Total 0.00 USD

Terms and Conditions

Terms and Conditions

1. A credit line will be prominently placed and include: for books - the author(s), title of book, editor, copyright holder, year of publication; For journals - the author(s), title of article, title of journal, volume number, issue number and inclusive pages.
2. The requestor warrants that the material shall not be used in any manner which may be considered derogatory to the title, content, or authors of the material, or to Wolters Kluwer/Lippincott, Williams & Wilkins.
3. Permission is granted for one time use only as specified in your correspondence. Rights herein do not apply to future reproductions, editions, revisions, or other derivative works. Once term has expired, permission to renew must be made in writing.
4. Permission granted is non-exclusive, and is valid throughout the world in the English language and the languages specified in your original request.
5. Wolters Kluwer Health/ Lippincott, Williams & Wilkins, cannot supply the requestor with the original artwork or a "clean copy."
6. The requestor agrees to secure written permission from the author (for book material only).
7. Permission is valid if the borrowed material is original to a LWW imprint (Lippincott-Raven Publishers, Williams & Wilkins, Lea & Febiger, Harwal, Igaku-Shoin, Rapid Science, Little Brown & Company, Harper & Row Medical, American Journal of Nursing Co, and Urban & Schwarzenberg - English Language).
8. If you opt not to use the material requested above, please notify Rightslink within 90 days of the original invoice date.

v1.0

WOLTERS KLUWER HEALTH LICENSE
TERMS AND CONDITIONS

Feb 27, 2009

This is a License Agreement between Andrew M Jackson ("You") and Wolters Kluwer Health ("Wolters Kluwer Health") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Wolters Kluwer Health, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	2137261141571
License date	Feb 27, 2009
Licensed content publisher	Wolters Kluwer Health
Licensed content publication	Transplantation
Licensed content title	Current Status of Clinical Islet Transplantation.
Licensed content author	Korsgren, Olle
Licensed content date	Jan 1, 2005
Volume Number	79
Issue Number	10
Type of Use	Dissertation/Thesis
Requestor type	Individual
Order reference number	korsgren table
Title of your thesis / dissertation	Analysis of Inflammatory responses in human pancreatic islet cells
Expected completion date	May 2009
Estimated size(pages)	110
Billing Type	Invoice
Billing Address	4210 Wild Plum Dr Carrollton, TX 75010

United States

Customer reference info

Total 0.00 USD

Terms and Conditions

Terms and Conditions

1. A credit line will be prominently placed and include: for books - the author(s), title of book, editor, copyright holder, year of publication; For journals - the author(s), title of article, title of journal, volume number, issue number and inclusive pages.
2. The requestor warrants that the material shall not be used in any manner which may be considered derogatory to the title, content, or authors of the material, or to Wolters Kluwer/Lippincott, Williams & Wilkins.
3. Permission is granted for one time use only as specified in your correspondence. Rights herein do not apply to future reproductions, editions, revisions, or other derivative works. Once term has expired, permission to renew must be made in writing.
4. Permission granted is non-exclusive, and is valid throughout the world in the English language and the languages specified in your original request.
5. Wolters Kluwer Health/ Lippincott, Williams & Wilkins, cannot supply the requestor with the original artwork or a "clean copy."
6. The requestor agrees to secure written permission from the author (for book material only).
7. Permission is valid if the borrowed material is original to a LWW imprint (Lippincott-Raven Publishers, Williams & Wilkins, Lea & Febiger, Harwal, Igaku-Shoin, Rapid Science, Little Brown & Company, Harper & Row Medical, American Journal of Nursing Co, and Urban & Schwarzenberg - English Language).
8. If you opt not to use the material requested above, please notify Rightslink within 90 days of the original invoice date.

v1.0

BIBLIOGRAPHY

1. King H, Aubert RE, Herman WH. *Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections*. Diabetes Care 1998;21(9):1414-1431.

2. Association AD. *Economic cost of diabetes in the US in 2007*. Diabetes Care 2007;31(3):596-615.

3. Association AD. *Economic costs of Diabetes in the US in 2002*. Diabetes Care 2003;26(3):917-932.

4. Boyle JP, Honeycutt AA, Narayan KM, Hoerger TJ, Geiss LS, Chen H, et al. *Projection of diabetes burden through 2050: impact of changing demography and disease prevalence in the U.S*. Diabetes Care 2001;24(11):1936-1940.

5. Ryan EA, Shandro T, Green K, Paty BW, Senior PA, Bigam D, et al. *Assessment of the severity of hypoglycemia and glycemic lability in type 1 diabetic sujects undergoing islet transplantation*. Diabetes 2004;53(4):955-962.

6. Reece EA, Coustan DR, Gabbe SG. *The History of Diabetes Mellitus in Women*. *Diabetes in Women*. 3rd ed: Lippinocott Williams & Wilkins; 2004.

7. Nabipour I. *Clinical endocrinology in the Islamic civilization in Iran*. Int. J. Endocrin. Metab. 2003;1:43-45.

8. Dobson M. *Nature of the urine in diabetes*. Med. Obs. Inquir 1776;5:298-310.

9. Von Mering J, Minkowski O. *Diabetes Mellitus naeh Pankreasexstirpation.* Arch. Exp. Pathol. Pharmakol 1890;26:371-387.

10. Williams PW. *Notes on diabetes treated with extract and by grafts of sheep's pancreas*. Br. Med. J. 1894;2(1771):1303-1304.

11. Banting FG, Best CH. *Pancreatic extracts.* . J. Lab. Clin. Med. 1990;115(2):254-272.

12. Banting FG, Best CH, Collip JB, Campbell WR, Fletcher AA. *Pancreatic extracts in the treatment of diabetes mellitus*. CMAJ 1991;145(10):1281-1286.

13. Banting FG, Campbell WR, Fletcher AA. *Further clinical experience with insulin (pancreatic extracts) in the treatment of diabetes mellitus*. Br. Med. J. 1923;1(3236):8-12.

14. Gaglia JL, Shapiro AMJ, Weir GC. *Islet transplantation: Progress and Challenge.* Arch. Of Med. Res. 2005;36(3):273-280.

15. Teuscher A. The History of Insulin. Insulin-A Voice for Choice. Basel: Karger; 2007.

16. Kelly WD, Lillehei RC, Merkel FK, Idezuki Y, Goetz FC. *Allotransplantation of the pancreas and duodenum along with the kidney in diabetic nephropathy*. Surgery 1967;61(6):827-837.

17. Teixeira E, Monteiro G, De Cenzo M, Teixeira A, Bergan JJ. *Transplantation of the isolated pancreas: report on the first human case*. Bull. Soc. Int. Chir 1970;29(6):337-344.

18. Ballinger WF, Lacy PE. *Transplantation of intact pancreatic islets in rats.* Surgery 1972;72(2):175-186.

19. Trucco M. *Regeneration of the Pancreatic β Cell.* J. Clin. Invest. 2005;115(1):5-12.

20. Cabrera O, Berman DM, Kenyon NS, Ricordi C, Berggren PO, Caicedo A. *The Unique Cytoarchitecture of Human Pancreatic Islets has Implications for Islet Cell Function.* Proc. Natl. Acad. Sci. USA. 2006;103(7):2334-2339.

21. Korsgren O, Nilsson B, Berne C, Felldin M, Foss A, Kallan R, et al. *Current Status of Clinical Islet Cell Transplantation.* . Transplantation 2005;79(10):1289-1293.

22. Knip M, Siljander H. *Autoimmune mechanisms in type 1 diabetes*. Autoimmun. Rev 2008;7(7):550-557.

23. Bottazzo GF, Florin-Christensen A, Doniach D. *Islet cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies.* Lancet 1974;304(7892):1279-1283.

24. Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK, et al. *Insulin antibodies in insulin-dependent diabetics before insulin treatment*. Science 1983;222(4630):1337-1339.

25. Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Soimena M, Cascalho M, et al. *Identification of the 64k autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase.* Nature 1990;347(6289):151-156.

26. Bonifacio E, Lampasona V, Genovese S, Ferrari M, Bosi E. *Identification of protein tyrosine phosphatase-like IA-2 (islet cell antigen 512) as the insulin-dependent diabetes-related 37/40k autoantigen and a target of islet-cell antibodies.* J. Immunol 1995;155(11):5419-5426.

27. Rabin DU, Pleasic SM, Shapiro JA, Yoo-Warren H, Oles J, Hicks JM, et al. *Islet cell antigen 512 is a diabetes-specific islet autoantigen related to protein tyrosine phosphatases*. J. Immunol. 1994;152(6):3183-3188.

28. Nayak RC, Omar MAK, Rabizadeh A, Srikanta S, Eisenbarth GS. *“Cytoplasmic” islet cell antibodies. Evidence that the target antigen is a sialogglycoconjugate.* Diabetes 1985;34(6):617-619.

29. Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, et al. *The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes*. Proc. Natl. Acad. Sci. USA. 2007;104(43):17040-17045.

30. Mansson L, Torn C, Landin-Olsson M. *Islet cell antibodies represent autoimmune response against several antigens.* Int. J. Exp. Diabetes Res. 2001;2(2):85-90.

31. Thorsby E, Ronningen KS. *Particular HLA-DQ molecules play a dominant role in determining susceptibility or resistance to type 1 (insulin-dependent) diabetes mellitus.* Diabetologia 1993;36(5):371-377.

32. Ilonen J, Reijonen H, Herva E, Sjoroos M, Iitia A, Lovgren T, et al. *Rapid HLA-DQB1 geneotyping for four alleles in the assessment of risk for IDDM in the finnish population.* Diabetes Care 1996;19(8):795-800.

33. Kimpimaki T, Kupila A, Hamalainen AM, Kukko M, Kulmala P, Savkola K, et al. *The first signs of β-cell autoimmunity appear in infancy in genetically susceptible children from the general population: the finnish type 1 diabetes prediction and prevention study.* J. Clin. Endocrinol Metab. 2001;86(10):4782-4788.

34. Knip M, Kukko M, Kulmala P, Veijola R, Simell O, Akerblom HK, et al. *Humoral beta-cell autoimmunity in relation to HLA-defined disease susceptibility in preclinical and clinical type 1 diabetes.* Am. J. Med. Genet. 2001;115(1):45-54.

35. Mannering SI, Harrison LC, Williamson NA, Morris JS, Thearle DJ, Jensen KP, et al. *The insulin A-chain epitope recognized by human T cells is posttranslationally modified.* J. Exp. Med. 2005;202(9):1191-1197.

36. Rabinovitch A, Suarez-Pinzon WL. *Roles of cytokines in the pathogenesis and therapy of type 1 diabetes.* Cell Biochem. Biophys. 2007;48(2-3):159-163.

37. Campbell PD, Estella E, Dudek NL, Jhala G, Thomas HE, Kay TWH, et al. *Cytotoxic t-lymphocyte-mediated killing of human pancreatic islet cells in vitro.* Hum. Immunol. 2008;69(9):543-551.

38. Lilleher RC, Idezuki Y, Dietzman RH, Kelly WD, Merkel FK, Goetz FC, et al. *Transplantation of the stomach, intestine, and pancreas: experimental and clinical observations*. Surgery 1967;62(4):721-741.

39. Mai ML, Ahsan N, Gonwa T. *The Long-term management of pancreas transplantation.* Transplantation 2006;82(8):991-1003.

40. Lipshutz GS, Wilkinson AH. *Pancreas-Kidney and Pancreas transplantation of the treatment of Diabetes Mellitus.* Endocrinol. Metab. Clin. North Am. 2007;36(4):1015-1038.

41. Feng S, Barr M, Roberts J, Oberbauer R, Kaplan B. *Developments in clinical islet, liver thoracic, kidney and pancreas transplantation in the last 5 years.* Am. J. Transplant. 2006;6(8):1759-1767.

42. Sutherland DE, Matas AJ, Goetz FC, Najarain JS. *Transplantation of dispersed islet tissue in humans: autografts and allografts*. Diabetes 1980;29(suppl 1):31-44.

43. 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 1973;16(6):686-689.

44. Horaguchi A, Merrel RC. *Preparation of viable islet cells from dogs by a new method.* Diabetes 1981;30(5):455-458.

45. Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW. *Automated method for isolation of human pancreatic islets.* Diabetes 1988;37(4):413-420.

46. Shapiro AMJ, Lakey JRT, Ryan EA, Korbutt GS, Toth E, Warnock GL, et al. *Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen.* N. Engl. J. Med. 2000;343(4):230-238.

47. Zeng YC, Ricordi C, Lendoire J, Carroll PB, Alejandro R, Bereiter DR, et al. *The effect of prednisone on pancreatic islet autografts in dogs.* Surgery 1993;113(1):98-102.

48. Brendel MD, Hering B, Schultz A, Bretzel R. *International Islet Transplant Registry Report.* Giessen: University of Giessen.; 1999. 1-20 p.

49. Bretzel RG. *Current status and perspectives in clinical islet transplantation.* J. Hepatobiliary Pancreat. Surg. 2000;7(4):370-373.

50. Shapiro AMJ, Ricordi C, Hering B. *Edmonton's islet success has indeed been replicated elsewhere.* Lancet 2003;362(9391):1242.

51. Shapiro AMJ, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, et al. *International trial of the Edmonton protocol for islet transplantation.* N. Engl. J. Med. 2006;355(13):1318-1330.

52. Hering BJ, Kandaswamy R, Ansite JD, Eckman PM, Nakano M, Sawada T, et al. *Single-Donor, Marginal -Dose Islet Transplantation in Patients with Type 1 Diabetes.* JAMA 2005;293(7):830-835.

53. Matsumoto S, Okitsu T, Iwanaga Y, Noguchi H, Nagata H, Yonekawa Y, Yamada Y, Fukuda K, et al. *Successful Islet Transplantation from Nonheartbeating Donor Pancreata Using Modified Ricordi Islet Isolation Method.* Transplantation 2006;82(4):460-465.

54. Ricordi C, Fraker C, Szust J, Al-Abdullah I, Poggioli R, Kirlew T, et al. *Improved human islet isolation outcome from marginal donors following addition of oxygenated perfluorocarbon to the cold storage solution.* Transplantation 2003;75(9):1524-1527.

55. Ryan EA, Lakey JR, Paty BW, Imes S, Korbutt GS, Kneteman NM, et al. *Successful islet transplantation: continued insulin reserve provides long-term glycemic control.* Diabetes 2002;51(7):2148-2157.

56. Beattie GM, Montgomery AMP, Lopez AD, Hao E, Perez B, Just ML, et al. *A novel approach to increase human islet cell mass while preserving β-cell function.* Diabetes 2002;51(12):3435-3439.

57. Ichii H, Wang X, Messinger S, Alvarez A, Fraker C, Khan A, et al. *Improved human islet isolation using nicotinamide.* Am. J. Transplant. 2006;6(9):2060-2068.

58. Biancone L, Cantaluppi V, Romanazzi GM, Russo S, Figliolini F, Beltramo S, et al. *Platelet-Activating factor sysnthesis and response on pancreatic islet endothelial cells: relevance for islet transplantation.* Transplantation 2006;81(4):511-518.

59. Kapturczak M, Zolotukhin S, Cross J, Pileggi A, Molano RD, Jorgensen M, et al. *Transduction of human and mouse pancreatic islet cells using a bicistronic recombinant adeno-associated viral vector.* Mol. Ther. 2002;5(2):154-160.

60. Klein D, Ribeiro MM, Mendoza V, Jayaraman S, Kenyon NS, Pileggi A, et al. *Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets.* Biochem. Biophys. Res. Comm. 2004;323(2):473-478.

61. Fenjves ES, Ochoa MS, Gay-Rabinstein C, Molano RD, Pileggi A, Mendez AJ, et al. *Adenoviral gene transfer of erythropoietin confers cytoprotection to isolated pancreatic islets.* Transplantation 2004;77(1):13-18.

62. Bennet W, Sundberg B, Groth CG, Brendel MD, Brandhorst D, Brandhorst H, et al. *Incompatiblilty Between Human Blood and Isolated Islets of Langerhans: a Finding with Implications for Clinical Intraportal Islet Transplantation?* Diabetes 1999;48(10):1907-1914.

63. Johansson H, Lukinius A, Moberg L, Lundgren T, Berne C, Foss A, et al. *Tissue factor produced by the endocrine cells of the islets of langerhans is associated with a negative outcome of clinical islet transplantation.* Diabetes 2006;54(6):1755-1762.

64. Emanmaullee J, Rajotte R, Liston P, Korneluk RG, Lakey JR, Shapiro AMJ, et al. *XIAP overexpression in human islets prevents early posttransplant apoptosis and reduces the islet mass needed to treat diabetes.* Diabetes 2005;54(9):2541-2548.

65. Kaplan B, West P, Neeley H, Martelloto J, Igbal R, Gangemi A, Hatipoglu B, Benedetti E, Oberholzer J. *Use of low dose tacrolimus, mycophenolate mofetil and maintenance IL-2 receptor blockade in an islet transplant recipient.* Clin. Transplant. 2008;22(2):250-253.

66. Zhang N, Su D, Qu S, Tse T, Bottino R, Balamurugan AN, Xu J, Bromberg JS, Dong HH. *Sirolimus is associated with reduced islet engraftment and impaired beta-cell function.* Diabetes 2006;55(9):2429-2436.

67. McDaniel ML, Marshall CA, Pappan KL, Kwon G. *Metabolic and autocrine regulation of the mammalian target of rapamycin by pancreatic beta-cells.* Diabetes 2002;51(10):2877-2885.

68. Larsen JL, Bennet RG, Burkman T, Ramirez AL, Yamamoto S, Gulizia J, Radio S, Hamel FG. *Tacrolimus and sirolimus cause insulin resistance in normal sprague dawley rats.* Transplantation 2006;82(4):466-470.

69. Cross SE, Richards SK, Clark A, Benest AV, Bates DO, Mathieson PW, Johnson PR, Harper SJ, Smith RM. *Vascular endothelial growth factor as a survival factor for human islets: effect of immunosuppressive drugs.* Diabetologia 2007;50(7):1423-1432.

70. Aronovitz A, Josefson J, Fisher A, Newman M, Hughes E, Chen F, Moons DS, Kiyokawa H, Jr. LWL. *Rapamycin inhibits growth factor-induced cell cycle regulation in pancreatic beta cells.* J. Investig. Med. 2008;56(8):985-996.

71. Bussiere CT, Lakey JR, Shapiro AMJ, Korbutt GS. *The impact of the mTOR inhibitor sirolimus on the proliferation and function of pancreatic islets and ductal cells.* Diabetologia 2006;49(10):2341-2349.

72. Froud T, Baidal DA, Ponte G, Ferreira JV, Ricordi C, Alejandro R. *Resolution of neurotoxicity and beta-cell toxicity in an islet transplant recipient following substitution of tacrolimus with MMF.* Cell Transplant. 2006;15(7):613-620.

73. Redmon JB, Olson LK, Armstron MB, Greene MJ, Robertson RP. *Effects of tacrolimus (FK506) on human insulin gene expression, insulin mRNA levels, and insulin secretion in HIT-T15 cells.* J. Clin. Invest. 1996;98(12):2786-2793.

74. Zeng XX, Sanchez-Fueyo A, Sho M, Domening C, Sayegh MH, Strom TB. *Favorably tipping the balance between cytopathic and regulatory T cells to create transplantation tolerance.* Immunity 2003;19(4):503-514.

75. Ricordi C, Strom TB. *Clinical islet transplantation: advances and immunological challenges.* Nat. Rev. Immunol. 2004;4(4):259-268.

76. Lobo PI, Spencer C, Simmons WD, Hagspiel KD, Angle JF, Markmann J, et al. *Development of anti-human leukocyte antigen class 1 antibodies following allogenic islet cell transplantation*. Transplant. Proc. 2005;37(8):3438-3440.

77. Campbell PM, Senior PA, Salam A, LaBranche K, Bigam DL, Kneteman NM, et al. *High Risk of Sensitization After Failed Islet Transplantation.* Am. J. Transplant. 2007;7(10):2311-2317.

78. Rickels MR, Kamoun M, Kearns J, Markmann JF, Naji A. *Evidence for allograft rejection in an islet transplant recipient and effect on β-cell secretory capacity.* J. Clin. Endocrin. and Metab. 2007;92(7):2410-2414.

79. Mohanakumar T, Narayanan K, Desai N, Ramachandran S, Shenoy S, Jendrisak M, et al. *A significant role for histocompatibility in human islet transplantation.* Transplantation 2006;82(2):180-187.

80. Campbell PM, Salam A, Ryan EA, Senior P, Paty BW, Bigam D, et al. *Pretransplant HLA antibodies are associated with reduced graft survival after clinical islet transplantation.* Am. J. Transplant. 2007;7(5):1242-1248.

81. Olack BJ, Swanson CJ, Flavin KS, Phelan D, Brennan DC, White NH, et al. *Sensitization to HLA antigens in islet recipients with failing transplants.* Transplant. Proc. 1997;29(4):2268-2269.

82. Tsujimura T, Kuroda Y, Avila JG, Kin T, Oberholzer J, Shapiro AMJ, et al. *Influence of Pancreas Preservation on Human Islet Isolation Outcomes: Impact of the Two-Layer Method.* Transplantation 2004;78(1):96-100.

83. Pujol-Borrell R, Hanafusa T, Chiovato L, et al. *Lectin-induced expression of DR antigen on human cultured follicular thyroid cells.* Nature 1983;304(5921):71-73.

84. Pujol-Borrell R, Todd I, Doshi M, Bottazzo GF, Sutton R, Gray D, et al. *HLA class II induction in human islet cells by interferon-γ plus tumor necrosis factor or lymphotoxin.* Nature 1987;326(6110):304-306.

85. Kim KA, Kim S, Chang I, Kim GS, Min YK, Lee MK, et al. *IFNγ/TNFα synergism in MHC class II induction: effect of nicotinamide on MHC class II expression but not on islet-cell apoptosis.* Diabetologia 2002;45(3):385-393.

86. Ricordi C, Gray DW, Hering BJ, Kaufman DB, Warnock GL, Kneteman NM, et al. *Islet Isolation Assessment in Man and Large Animals.* Acta Diabletol. Lat. 1990;27(3):185-195.

87. Witkowski P, Lui Z, Guo Q, Poumian-Ruiz E, Cernea S, Herold K, et al. *Two-Layer Method in Short-Term Pancreas Preservation for Successful Islet Isolation.* Transplant. Proc. 2005;37(8):3398-3401.

88. Tsujimura T, Kuroda Y, Churchill TA, Avila JG, Kin T, Shapiro AMJ, et al. *Short-Term Storage of the Ischemically Damaged Human Pancreas by the Two-Layer Method Prior to Islet Isolation*. Cell Transplant. 2004;13(1):67-73.

89. Matsumoto S, Qualley SA, Goel S, Hagman DK, Sweet IR, Poitout V, Strong DM, Robertson RP, Reems JA. *Effect of the two-layer (University of Wisconsin solution–perfluorochemical plus O2) method of pancreas preservation on human islet isolation, as assessed by the Edmonton Isolation Protocol.* Transplantation 2002;74(10):1414-1419.

90. Cabellero-Corbalan J, Eich T, Lundgren T, Foss A, Felldin M, Kallan R, et al. *No Beneficial Effect of Two-Layer Storage Compared with UW-Storage on Human Islet Isolation and Transplantation.* Transplantation 2007;84(7):864-869.

91. Sabek OM, Cowan P, Fraga DW, Gaber AO. *The Effect of Isolation Methods and the use of Different Enzymes on Islet Yield and in Vivo Function.* Cell Transplant. 2008;17(7):785-792.

92. Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, et al. *Five-Year Follow-up After Clinical Islet Transplantation.* Diabetes 2005;54(7):2060-2069.

93. Ozmen L, Ekdahl KN, Elgue G, Larsson R, Korsgren O, Nilsson B. *Inhibition of Trombin Abrogates the Instant Blood-Mediated Inflammatory Reaction Triggered by Isolated Human Islets.* Diabetes 2002;51(6):1779-1784.

94. Moberg L, Johansson H, Lukinius A, Berne C, Foss A, Kallen R, et al. *Production of Tissue Factor by Pancreatic Islet Cells as a Trigger of Detrimental Thrombotic Reactions in Clinical Islet Transplantation.* Lancet 2002;360(9350):2039-2045.

95. Moberg L, Olsson A, Berne C, Felldin M, Foss A, Kallen R, et al. *Nicotinamide Inhibits Tissue Factor Expression in Isolated Human Pancreatic Islets: Implications for Clinical Islet Transplantation.* Transplantation 2003;76(9):1285-1288.

96. Goto M, Johansson H, Maeda A, Elgue G, Korsgren O, Nilsson B. *Low-Molecular Weight Dextran Sulfate Abrogates the Instant Blood-Mediated Inflammatory Reaction Induced by Adult Porcine Islets both in Vitro and in Vivo.* Transplant. Proc. 2004;36(4):1186-1187.

97. 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 2004;77(5):741-747.

98. Moberg L, Korsgren O, Nilsson B. *Neutrophilic Granulocytes are the Predominant Cell Type Infiltrating Pancreatic Islets in Contact with ABO-Compatible Blood.* Clin. Exp. Immun. 2005;142(1):125-131.

99. Johansson H, Goto M, Siegbahn A, Elgue G, Korsgren O, Nilsson B. *Low Molecular Weight Dextran Sulfate: A Strong Candidate Drug to Block IBMIR in Clinical Islet Transplantation.* Am. J. Transplant. 2006;6(2):305-312.

100. Tjernberg J, Ekdahl KN, Lambris JD, Korsgren O, Nilsson B. *Acute Antibody-Mediated Complement Activation Mediates Lysis of Pancreatic Islet Cells and May Cause Tissue Loss in Clinical Islet Transplantation.* Transplantation 2008;85(8):1193-1199.

101. Jackson A, McWilliams C, Kaizer E, Chaussabel D, Glaser C, Noguchi H, et al. *Gene Expression Profiling of Human Pancreatic Islets Undergoing a Simulated Process of Instant Blood-Mediated Inflammatory Reaction.* Transplant. Proc. 2008;40(2):430-432.

102. Matsumoto S, Rigley TH, Qualley SA, Kuroda Y, Reems JA, Stevens RB. *Efficacy of the oxygen-charged static two-layer method for short-term pancreas preservation and islet isolation from nonhuman primate and human pancreata.* Cell Transplant. 2002;11(8):769-777.

103. Goto M, Tjernberg J, Dufrane D, Elgue G, Brandhorst D, Ekdahl KN, et al. *Dissecting the Instant Blood-Mediated Inflammatory Reaction in Islet Xenotransplantation.* Xenotransplantation 2008;15(4):225-234.

104. Contreras JL, Eckstein C, Smyth CA, Sellers MT, Vilatoba M, Bilbao G, et al. *Brain Death Significantly Reduces Isolated Pancreatic Islet Yields and Functionality in Vitro and in Vivo After Transplantation in Rats.* Diabetes 2003;52(12):2935-2942.

105. Kaizer EC, Glaser CL, Chaussabel D, Banchereau J, Pascual V, White PC. Gene Expression in Peripheral Blood Mononuclear Cells in Children with Diabetes. J. Clin. Endocrinol. Metab. 2007;92(9):3705-3711.

106. Osman M, Tortorella M, Londei M, Quarantino S. *Expression of Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinases Define the Migratory Characteristics of Human Monocyte-Derived Dendritic Cells.* Immunology 2002;105(1):73-82.

107. Barro C, Zaoui P, Morel F, Benhamou PY. *Matrix Metalloproteinase Expression in Rat Pancreatic Islets.* Pancreas 1998;17(4):378-382.

108. Yu Q, Stamenkovic I. *Cell Surface-Localized Matrix Metalloproteinase-9 Proteolytically activates TGF-β and Promotes Tumor Invasion and Angiogenesis.* Genes Dev. 2000;14(2):163-176.

109. Olsson R, Maxhuni A, Carlsson PO. *Revascularization of Transplanted Pancreatic Islets Following Culture with Stimulators of Angiogenesis.* Transplantation 2006;82(3):340-347.

110. Voronov E, Shouval DS, Krelin Y, Cagnano E, Benharroch D, Iwakura Y, et al. *IL-1 is Required for Tumor Invasiveness and Angiogenesis.* Proc. Natl. Acad. Sci. USA. 2003;100(5):2645-2650.

111. Baek SH, Ohgi KA, Rose DW, Koo EH, Glass CK, Rosenfeld MG. *Exchange of N-CoR Corepressor and Tip60 Coactivator Complexes Links Gene Expression by NF-κB and β-Amyloid Precursor Protein.* Cell 2002;110(1):55-67.

112. Ortis F, Naamane N, Kreins AY, Rasschaert J, Moore F, Theatre E, et al. *Induction of Nuclear Factor-κB and its Downstream Genes by TNF-α and IL-1β has a Pro-Apoptotic Role in Pancreatic Beta Cells.* Diabetologia 2008;51(7):1213-1225.

113. Mandrup-Poulsen T. *The Role of Interleukin-1 in the Pathogenesis of IDDM.* Diabetologia 1996;39(9):1005-1029.

114. Paul SR, Bennet F, Calvetti JA, Kelleher K, Wood CR, O'Hara RM. *Molecular Cloning of a cDNA Encoding Interleukin 11, a Stromal Cell-Derived Lymphopoietic and Hematopoietic Cytokine.* Proc. Natl. Acad. Sci. USA. 1990;87(19):7512-7516.

115. Du XX, Williams DA. *Interleukin-11: A Multifunctional Growth Factor Derived From the Hematopoietic Microenvironment.* Blood 1994;83(8):2023-2030.

116. Maier R, Ganu V, Lotz M. *Interleukin-11, an Inducible Cytokine in Human Articular Chondrocytes and Synoviocytes, Stimulates the Production of the Tissue Inhibitor of Metalloproteinases.* J. Biol. Chem. 1993;268(29):21527-21532.

117. Oppman B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, et al. *Novel p19 Protein Engages IL-12p40 to Form a Cytokine, IL-23, with Biological Activities Similar as Well as Distinct from IL-12.* Immunity 2000;13(5):715-725.

118. Langowski JL, Zhang X, Wu L, Mattson JD, Chen T, Smith K, et al. *IL-23 Promotes tumor Incidence and Growth.* Nature 2006;442(27):461-465.

119. Schwarz A, Stander S, Berneburg M, Bohm M, Kulms D, van Steeg H, et al. *Interleukin-12 Suppresses Ultraviolet Radiation-Induced Apoptosis by inducing DNA Repair.* Nat. Cell Biol. 2002;4(1):26-31.

120. Ferlazzo G, Thomas D, Lin SL, Goodman K, Morandi B, Muller WA, et al. *The Abundant NK Cells in Human Secondary Lymphoid Tissues Require Activation to Express Killer Cell Ig-Like Receptors and Become Cytolytic.* J. Immunol. 2004;172(3):1455-1462.

121. Niedbala W, Wei XQ, Cai B, Hueber AJ, Leung BP, McInnes IB, et al. *IL-35 is a Novel Cytokine With Therapeutic Effects Against Collagen-Induced Arthritis Through the Expansion of Regulatory T Cells and the Suppression of Th17 Cells.* Eur. J. Immunol. 2007;37(11):3021-3029.

122. Collison LW, Workman CG, Kuo TT, Boyd K, Wang Y, Vignali KM, et al. *The Inhibitory Cytokine IL-35 Contributes to Regulatory T-Cell Function.* Nature 2007;450(22):566-569.

123. Reynet C, Kahn CR. *Rad: A Member of the Ras Family Overexpressed in Muscle of Type II Diabetic Humans.* Science 1993;262(5138):1441-1444.

124. Vojtek AB, Der CJ. *Increasing Complexity of the Ras Signaling Pathway.* J. Biol. Chem. 1998;273(32):19925-19928.

125. Kauffmann-Zeh A, Rodriguez-Viciana P, Ulrich E, Gilbert C, Coffer P, Downward J, et al. *Suppression of c-Myc-Induced Apoptosis by Ras Signaling Through PI9(3)K and PKB.* Nature 1997;385(6616):544-548.

126. Yang G, Rosen DG, Zhang Z, Bast RC, Mills GB, Colacino AC, et al. *The Chemokine Growth-Regulated Oncogene 1 (Gro-1) Links RAS Signaling to the Senescence of Stromal Fibroblasts and Ovarian Tumorigenesis.* Proc. Natl. Acad. Sci. USA. 2006;103(44):16472-16477.

127. Mohsenin A, Burdick MD, Molina JG, Keane MP, Blackburn MR. *Enhanced CXCL1 Production and Angiogenesis in Adenosine-Mediated Lung Disease.* FASEB J. 2007;21(4):1026-1036.

128. Masters BA, Kelly EJ, Quaife CJ, Brinster RL, Palmiter RD. *Targeted Disruption of Metallothionein I and II Genes Increases Sensitivity to Cadmium.* Proc. Natl. Acad. Sci. USA. 1994;91(2):584-588.

129. Hou N, Torii S, Saito N, Hosaka M, Takeuchi T. *Reactive Oxygen Species-Mediated Pancreatic Beta-Cell Death is Regulated by Interactions Between Stress-Activated Protein Kinases, p38 and c-Jun N-Terminal Kinase, and Mitogen-Activated Protein Kinase Phosphatases.* Endocrinology 2008;149(4):1654-1665.

130. Maedler K, Sergeev P, Ehses JA, Mathe Z, Bosco D, Berney T, et al. Leptin Modulates β Cell Expression of IL-1 Receptor Antagonist and Release of IL-1β in Human Islets. Proc. Natl. Acad. Sci. USA. 2004;101(21):8138-8143.

131. Larsen CM, Faulenbach M, Vaag A, Volund A, Ehses JA, Seifert B, et al. *Interleukin-1-Receptor Antagonist in Type 2 Diabetes Mellitus.* N. Engl. J. Med. 2007;356(15):1517-1526.

132. Jackson A, Connolly JE, Matsumoto S, Noguchi H, Onaca N, Levy MF, Naziruddin B. Evidence for Induced HLA class II Expression. Transplantation 2009;87(4):500-506.

133. Soldevila G, Boscema M, Doshi M, James RFL, Bottazzo GF, Pujol-Borrell R. *Cytotoxic effect of IFN gamma plus TNF alpha on human islet cells.* J. Autoimmun. 1991;4(2):291-306.

134. Janeway CA, Travers P, Walport M, Shlomchik M, editors. Immunobiology. 6 ed. New York: Garland Science; 2005.

135. Chidgey AP, Layton D, Trounson A, Boyd RL. *Tolerance Strategies for Stem-Cell-Based Therapies.* Nature 2008;453(7193):330-337.

136. Ichii H, Inveradi L, Pileggi A, Molano RD, Cabrera O, Caicedo A, et al. *A novel method for the assessment of composition and beta cell viability in human islet preparations.* Am. J. Transplant. 2005;5(7):1635-1645.

137. Chomczynski P, Sacchi N. *Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.* Anal. Biochem. 1987;162(1):156-159.

138. Foulis AK, Farquharson MA. *Aberant expression of HLA DR antigens in insulin containing beta cells in recent onset type 1 (insulin-dependent) diabetes mellitus.* Diabetes 1986;35(11):1215-1224.

139. Bottazzo GF, Dean BM, McNally JM, MacKay EH, Swift PG, Gamble DR. *In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis.* N. Engl. J. Med. 1985;313(6):353-360.

140. Pujol-Borrell R, Todd I, Doshi M, Gray D, Feldmann M, Bottazzo GF. *Differential expression and regulation of MHC products in the endocrine and exocrine cells of the human pancreas.* Clin. Exp. Immunol. 1986;65(1):128-139.

141. Campbell IL, Bizilj K, Colman PG, Tuch BE, Harrison LC. *Interferon-gamma induces the expression of HLA-A,B,C but not HLA-DR on human pancreatic beta-cells.* J. Clin. Endocrin. Metab. 1986;62(6):1101-1109.

142. Barshes NR, Wyllie S, Goss JA. *Inflammation-mediated dysfunction and apoptosis in islet transplantation: Implications for intrahepatic grafts.* J. Leukoc. Biol. 2005;77(5):587-597.

143. Bottino R, Fernandez LA, Ricordi C, Lehmann R, Tsan MF, Oliver R, et al. *Transplantation of allogeneic islets of langerhans in the rat liver: effects of macrophage depletion on graft survival and microenvironment activation.* Diabetes 1998;47(3):316-323.

144. Cardani R, Pileggi A, Ricordi C, Gomez C, Baidal DC, Ponte GG, et al. *Allosensitization of Islet Allograft Recipients.* Transplantation 2007;84(11):1413-1427.

145. Lui C, Noorchashm H, Sutter JA, Naji M, Prak EL, Boyer J, et al. *B-lymphocyte-directed immunotherapy promotes long-term islet allograft survival in nonhuman primates.* Nat. Med. 2007;13(11):1295-1298.

146. Caballero A, Fernandez N, Lavado R, Bravo MJ, Miranda JM, Alonso A. *Tolerogenic Response: Allorecognition Pathways*. Transpl. Immunol. 2006;17(1):3-6.

147. Benicchou G, Valujskikh A, Heeger PS. *Contributions of Direct and Indirect T Cell Alloreactivity During Allograft Rejection in Mice.* J. Immunol. 1999;162(1):352-358.

148. Auchincloss H, Lee R, Shea S, Markowitz JS, Grusby MJ, Glimcher LH. *The Role of "Indirect" recognition in Initiating Rejection of Skin Grafts from Major Histocompatibility Complex Class II-Deficient Mice.* Proc. Natl. Acad. Sci. USA. 1993;90(8):3373-3377.

149. Drenou B, Blancheteau V, Burgess DH, Fauchet R, Charron DJ, Mooney NA. *A caspase-independent pathway of MHC class II antigen-mediated apoptosis of human B lymphocytes.* J. Immunol. 1999;163(8):4115-4124.

150. Bertho N, Drenou B, Laupeze B, Le Berre C, Amiot L, Grosset JM, et al. *HLA-DR-mediated apoptosis susceptibility discriminates differentiation stages of dendritic/monocyte APC.* J. Immunol. 2000;164(5):2379-2385.

151. Kupfer TM, Crawford ML, Pham K, Gill RG. *MHC-Mismatched Islet Allografts are Vulnerable to Autoimmune Recognition in Vivo.* J. Immunol. 2005;175(4):2309-2316.

152. Chan WFN, Razavy H, Anderson CC. *Differential Susceptibility of Allogneic Targets to Indirect CD4 Immunity Generates Split Tolerance.* J. Immunol. 2008;181(7):4603-4612.

153. Mirenda V, Golshayan D, Read J, Berton I, Warrens AN, Dorling A, et al. *Achieving Permanent Survival of Islet Xenografts by Independent Manipulation of Direct and Indirect T-Cell Responses*. Diabetes 2005;54(4):1048-1055.

154. Dragun D. *Humoral Responses Directed Against Non-Human Leukocyte Antigens in Solid-Organ Transplantation*. Transplantation 2008;86(8):1019-1025.