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Prediction, Prevention and Treatment of Virally Induced Type 1 Diabetes: A Dissertation

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PREDICTION, PREVENTION AND TREATMENT OF VIRALLY INDUCED TYPE 1 DIABETES

A Dissertation Presented

By

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APPROVAL PAGE

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ABSTRACT

Several viral infections have been associated with human type 1 diabetes (T1D), although it has proven difficult to unequivocally establish them as causative agents. In rodent models, however, viruses have definitely been established to cause T1D. The treatment of weanling BBDR rats with the combination of a TLR3 ligand, pIC, and an ssDNA parvovirus, KRV, precipitates T1D in nearly 100% of rats within a short, predictable timeframe. In this dissertation, we utilized the BBDR rat model to (1) identify early serum biomarkers that could predict T1D precipitated by viral induction and (2) test the efficacy of leptin, a therapeutic agent, which may have the ability to prevent diabetes onset, reverse new onset diabetes and prevent autoimmune recurrence of diabetes in rats transplanted with syngeneic islet grafts.

Identification of biomarkers has long served as an invaluable tool for disease prediction. In BBDR rats, we identified an acute phase response protein, haptoglobin, as a potential biomarker for pIC + KRV induced T1D using the global proteomic profiling techniques, 2D gel analysis and iTRAQ. Upon validating this biomarker, we determined that haptoglobin was sensitive in predicting T1D in the pIC + KRV model, in which nearly 100% of the rats become diabetic, but not in models where diabetes expression was variable (KRV only or RCMV only models). However, analysis of the serum kinetics of haptoglobin and its functional capacity in the blood has given us insights into the potential role of early phase reactants in modulating virally mediated T1D.

An alternative means of regulating T1D pathogenesis is through leptin. Leptin is a hormone with pleotropic roles in the body, particularly affecting energy metabolism and

immune regulation. These characteristics make leptin an intriguing candidate for therapeutic testing in T1D models. Our studies have determined that high doses of leptin delivered via an adenovirus (AdLeptin) or alzet pump delivery system can prevent diabetes in > 90% of rats treated with pIC + KRV. We further showed that serum hyperleptinemia was associated with decreased body weight, decreased non-fasting serum insulin levels and lack of islet insulitis in pIC + KRV treated rats pretreated with AdLeptin compared with those pretreated with PBS. We discovered that hyperleptinemia induced a profound decrease in splenic weight and splenic cellularity, including reductions in CD4+ and CD8+ T cells, DC/MACs and B cells. These findings indicate a potential mechanism whereby hyperleptinemia protects rats from virally induced T1D through the promotion of peripheral immunosuppression.

Among pIC + KRV treated rats, we have also found that leptin therapy can reverse hyperglycemia in a subset of new onset diabetics for up to 20 days. In the absence of exogenous insulin, leptin treatment of new onset diabetics prevented the rapid weight loss associated with osmotic diuresis, as well as the ketosis observed in vehicle treated diabetic rats. Overall, these findings point to the therapeutic value of leptin in maintaining glycemic control and preventing ketosis in an insulin deficient state, in the absence of exogenous insulin therapy.

Additionally, we have also determined that AdLeptin treatment can prolong the survival of syngeneic islets transplanted into diabetic BBDR rats for up to 50 days post transplant. Although hyperleptinemia generated by AdLeptin was unable to prevent insulitis into islet grafts, this insulitis did not appear to be destructive as islet grafts continued to stain positively for insulin when compared with control rats whose grafts succumbed to recurrent

autoimmunity. In the various therapeutic settings in which we have tested leptin treatment, we have found this hormone to have significant beneficial effects. These findings merit further evaluation of leptin as a therapeutic agent in human T1D.

ABBREVIATIONS

.pkl	file extension for iTRAQ files
1D	one-dimensional
2D	two dimensional
Ab	antibody
ACN	acetonitrile
ACTH	adrenocorticotropic hormone
AdBetagal	adenovirus bearing the beta galactosidase gene construct
AdLeptin	adenovirus bearing the rat leptin gene construct
APC	antigen presenting cell
ATP	adenosine triphosphate
BBDR	biobreeding diabetes resistant
Bis-Tris	1,3-bis(tris(hydroxymethyl)methyl amino)propane
BME	beta mercaptoethanol
C-18	octadecyl type hydrocarbon with 18 carbon atoms
$CaCl_2$	calcium chloride
CBV	Coxsackie B viruses
CD	clusters of differentiation
cDMEM	Dulbecco's modified eagle medium
CFSE	carboxyfluorescein succinimidyl ester
CHAPS	3-[(3-Cholamidopropyl)dimethylam monio]-1-propanesulfonate
CID	collision-induced dissociation
CLN	cervical lymph nodes
CMV	cytomegalovirus
CNS	central nervous system
conA	concanavalin A
C-peptide	"connecting peptide"
CpG	cytosine-phosphate-guanine
CRH	corticotropin-releasing hormone
$CsCl_2$	cesium chloride
CTLA-4	cytotoxic T-Lymphocyte Antigen 4
Da	dalton
DAISY	Diabetes Autoimmunity Study of the Young
DC	dendritic cell
DC/MAC	dendritic cells and macrophages
DCCT	Diabetes Control and Complications Trial
DKA	diabetic ketoacidosis
DNA	deoxyribonucleic acid
DPT-1	Diabetes Prevention Trial
dsRNA	double stranded RNA
DTT	dithiothreitol
EAE	experimental allergic encephalomyelitis
EBI	European Bioinformatics Institute
ECL	electrochemiluminescence
ELISA	enzyme-linked immunosorbent assay
FA	formic acid
FACS	Fluorescence Activated Cell Sorting
FBS	fetal bovine serum
FFA	free fatty acid
GAD	glutamic acid decarboxylase
G-CSF	granulocyte-colony stimulating factor
H&E	hematoxilin and eosin
HbA1c	hemoglobin A1c
HBSS	Hank's balanced salt solution
HLA	human leukocyte antigen
HRP	horseradish peroxidase
icv	intracerebrovascular
i.p.	intraperitoneally
i.v.	intravenous
IA2	insulin autoantibody 2
ICA	islet cell antigen
ID	internal diameter
IEF	isoelectric focusing
IFN	interferon
IGF	insulin-like growth factors
IgG	immunoglobulin G

IGRP	islet-specific glucose-6-phosphatase catalytic subunit-related protein
IHC	immunohistochemistry
IL	interleukin
iNOS	inducible nitric oxide synthase
IPG	immobilized pH gradient
IPI	International Protein identification
iTRAQ	isobaric tag for relative and absolute quantitation
KCl	potassium chloride
kDa	kilodalton - unified atomic mass unit
KH_2PO4	mono potassium phosphate
KRV	Kilham rat virus
LC	liquid chromatography
LPK	rrLeptin + pIC + KRV
LPS	lipopolysaccharide
mAb	monoclonal antibody
MALDI	matrix-assisted-laser desorption/ionization
MDA	melanoma differentiation-associated gene
MHC	major histocompatibility class
MMTS	methyl methanethiosulfonate
MMTS	methyl methanethiosulfonate
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	mitochondrial RNA
MS	mass spectrometry
MW	molecular weight
NHS	TrialNet natural history study
NK	natural killer
NKRP	natural killer receptor protein
NKT	natural killer T
NOD	non-obese diabetic
NRK	normal rat kidney
NS	non-structural proteins
P14R	synthetic peptide (MW = 1533.86) for calibration
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PFUs	plaque forming units
pI	isoelectric point
pIC	polyinosinic:polycytidilic acid
PKR	protein kinase R
PLGS	ProteinLynx Global SERVER
PLN	pancreatic draining lymph node
PTPN22	protein tyrosine phosphatase, non-receptor type 22 (lymphoid)
PVDF	polyvinylidene fluoride
RBC	red blood cell
RCMV	rat cytomegalovirus
Reo-3	reovirus 3
RIA	radioimmunoassay
RNA	ribonucleic acid
RPM	revolutions per minute
RPMI	Roswell Park Memorial Institute
rrLeptin	rat recombinant leptin
RT6	ADP-ribosyltransferase
RTI	rat MHC class II haplotype
rVV	recombinant vaccinia virus
SCX	strong cation exchange
SDS	sodium dodecyl sulfate
SREBP	sterol regulatory element-binding protein
ssDNA	single stranded DNA
STZ	streptozodocin
T1D	Type 1 A diabetes mellitus
TBS	tris buffered saline
TCEP	Tris-(2-carboxyethyl) phosphine
TCR	T cell receptor
Teff	effector T cells
TFA	triflouroacetic acid
TG	triglyceride
TGF	transforming growth factor
Th	T helper
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
TOF	time of flight
Treg	regulatory T cells

Tris HCl	tris (hydroxymethyl)aminomethane plus concentrated hydrochloric acid
UC	ulcerative colitis
VP	viral capsid protein
VPK	vehicle + pIC + KRV
WF	wistar furth
ZnT	zinc transporter

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CHAPTER I: INTRODUCTION

Natural History of T1D in Humans

Disease Definition, Diagnosis and Impact

Diabetes mellitus is a suite of metabolic diseases that share a common phenotype: hyperglycemia. Type 1 A diabetes mellitus (T1D) results from the autoimmune destruction of beta cells in the pancreatic islets of langerhans. The clinical manifestations of hyperglycemia and T1D can be recognized by the appearance of the following symptoms: polyuria (frequent urination), polydipsia (excessive thirst), and polyphagia (excessive hunger). Commensurate with these symptoms, patients with T1D also experience fatigue, weight loss and weakness. The onset of disease is usually acute, developing over a period of a few days to weeks. The diagnosis of T1D is made when the symptoms of diabetes mentioned above are concomitant with positive findings from any two of the following tests on different days: (1) random blood glucose concentration greater than 200 mg/dL (2) fasting plasma glucose greater than 126 mg/dl or (3) a two hour plasma glucose greater than 200 mg/dL after a 75 g glucose load (oral glucose tolerance test). T1D affects between 700,000 to 1.4 million individuals in the United States today. The incidence rate of T1D in the United States is approximately 16 cases/100,000/year [1], and there is an equal incidence in both genders. About 40% of people with T1D develop the disease before 20 years of age, thus making it one of the most common severe chronic diseases of childhood [2].

Complications from T1D

Diabetic ketoacidosis (DKA) is the most acute, life threatening complication of T1D that relegates T1D patients to a life-long dependency on exogenous insulin. In the absence of beta cells, the body experiences an absolute insulin deficiency, a state that prevents many cells' ability to use a key fuel source, glucose. Decline in glucose utilization by peripheral tissues combined with excess of counter-regulatory hormones such as glucagon and norepinepherine prompts the liver to increase glucose production (via gluconeogenesis and glycogenolysis) as well as production of ketone bodies from free fatty acids released by adipocytes. In excess, the acidic nature of ketone bodies overwhelms the neutralizing capacity of serum bicarbonate levels and leads to metabolic ketoacidosis, a life threatening acid base disturbance. Rapid correction of the acid base disturbance, its concomitant electrolyte imbalance (especially serum potassium levels), as well as restoration of serum insulin levels are of paramount significance in saving patients' lives. Despite the availability of exogenous insulin therapy to treat T1D, the risk of DKA in patients remains high as it can be precipitated by increased requirement for insulin, for example, during a concurrent illness.

The administration of insulin, although lifesaving, can neither halt the persistent autoimmune response nor prevent some of the devastating long term complications of the disease, even when tight glycemic control is maintained [3]. These complications can result in diseases of the microvasculature, such as retinopathy, neuropathy or nephropathy, or diseases of the macrovasculature, including diseases such as coronary artery disease, peripheral vascular disease or cerebrovascular disease [4, 5]. Patients who develop T1D can additionally develop other autoimmune disorders such as Graves' disease, Hashimoto's

thyroiditis, Addison's disease, alopecia, vitiligo or pernicious anemia [5, 6]. Because insulin therapy has proven not to be the final cure for T1D, scientific progress in the identification of new, alternative therapies that can prevent T1D and its complications is essential.

Etiology of T1D Pathogenesis

The etiology of T1D is believed to be autoimmune in nature since autoreactive T cells and other inflammatory immune cells are found in the islets of type 1 diabetics, and autoantibodies to islets antigens have been found in the sera of both pre-diabetic and diabetic patients [7]. Additionally, T cells have been directly implicated in islet destruction in both human disease and rodent models. In fact, T cells specific for islet antigens GAD, IA2, insulin and glucose-6-phosphatase catalytic subunit-related protein (IGRP) are found in blood of diabetic and prediabetic patients [1]. Mechanisms leading to beta cell specific autoimmunity have not been entirely clarified, although more than one mechanism is believed to contribute to the expression of disease in a heterogeneous population. Overall, T1D is believed to be a polygenic disorder, its manifestation the consequence of a complex interplay between genetic and environmental factors.

Genetics

Many studies have reported T1D disease concordance in monozygotic twins to range between 30-50% [8]. One study recent reported concordance for T1D among monozygotic twins in a Finnish population to be 42.9% and 3% among dizygotic twins [9]. These investigators suggested an additive model for T1D risk with 88% of the phenotypic variance in T1D attributable to additive genetic effects and 12% due to environmental factors. Other

investigators have questioned the validity of low reported concordance estimates among monozygotic twins, arguing that the 30-50% range is based on ascertainment of data at a single time point rather than from long term follow-up of twins. Redondo and colleagues [10] followed monozygotic twins without diabetes prospectively for up to 43 years. They determined that among monozygotic twins initially discordant for the disease, by 60 years of age, 65% became concordant for disease, and 78% had persistent autoantibody levels, T1D or both. The investigators concluded that with long term follow-up of monozygotic twins, both autoantibody positivity and T1D develop more frequently than is currently estimated, especially in twins that may have been initially discordant for the disease. This study does attribute greater influence of genetic factors in T1D expression than previous studies. However, given that the concordance among monozygotic twins followed up for a long period is still not 100%, it is widely hypothesized that environmental perturbants may ultimately affect disease expression.

Major gene loci involved in susceptibility to T1D are located within the human leukocyte antigen (HLA) region on short arm of human chromosome 6. Genes in this loci represent 40-50% of the inheritable diabetes risk [11]. In Caucasians HLA Class II DR/DQ alleles (DR3, DR4, DQ8, DQ2) are associated with increased risk of diabetes development [12]. 30-50% of patients with T1D in this population are DR3/DR4 heterozygotes [13]. On the other hand, among the Japanese and most east Asian populations, DR4 and DR9 haplotypes have been linked with disease susceptibility [14]. Many non-HLA genes have also been associated with increased risk for diabetes in humans. These include the insulin gene, PTPN22 (a gene that encodes for a protein tyrosine phosphatase), CTLA-4 and CD25,

both involved in immunoregulation, and differentiation-associated gene-5 (MDA-5), an intracellular viral RNA detection molecule [1].

Environment

Although a host of HLA and non-HLA factors have been identified as contributing to the risk of T1D, the role of the environment in precipitating disease is a widely accepted hypothesis. Environmental triggers such as toxins, vaccinations, cow's milk protein, wheat protein, and viral infections have all been hypothesized to contribute to the etiology of T1D [15]. Further evidence implicating environmental factors in precipitating disease comes from migration studies. For example, investigators have found that children born in the UK to immigrants from Asian countries where the incidence of T1D is low show increased incidence of T1D compared with children in their parents' countries of origin [16, 17]. Geographic variation in disease also supports the role for environmental factors. Finland and Sweden, for example, suffer some of the highest incidence of T1D in the world, in stark contrast to the low incidence observed in many Asian and African countries [1].

Role of Viruses in T1D

Viral infections in particular have received significant attention as precipitating factors for T1D due to the mounting evidence associating infections with the onset of human disease. The only definitive casual association between viruses precipitating T1D exists for rubella infection. Nearly 20% of patients with congenital rubella have been documented to develop T1D [18, 19]. Other viruses that have been implicated in human T1D include enteroviruses such as Coxsackie B viruses (CBV), mumps, cytomegalovirus (CMV),

Epstein-Barr virus, varicella zoster virus, and rotavirus [1]. Lending weight to the hypothesis of virally mediated disease in humans is the existence of various rodent models where viruses have been shown to definitively precipitate the disease [20, 21]. It is difficult to establish viral causation of T1D in humans, however, because not all T1D cases are immediately preceded by viral infections. In fact, there is a high probability that by the time T1D is diagnosed the patient has been exposed to multiple viruses, and the causative viral infection has been cleared [1].

More recently, evidence has centered around enteroviral infections as causative agents of T1D. While enteroviruses have not been confirmed as etiologic agents in T1D, they are one of the most investigated pathogens with respective to human disease, as they are commonly found to accompany or precede T1D development [7]. Earlier studies have documented the occurrence of enterovirus infections more frequently in siblings that develop clinical T1D compared with non-diabetic siblings [22]. Other studies have found enteroviral RNA readily detectable in the blood from patients at diabetes onset [23, 24]. However, the lack of a strong clinical association between CBV infection and T1D in European countries where the prevalence of disease is very high has relegated the evidence for the causative role of enteroviruses in T1D as circumstantial [25]. In March 2009, a team of investigators revitalized the debate over the role of enteroviruses as causative agents. They provided new, clear evidence that enteroviruses are found frequently in the pancreas of people who develop diabetes. Their study assessed the prevalence of enteroviral capsid protein vp1 staining in a large cohort of autopsy pancreata of recent onset type 1 diabetic patients [26]. These investigators not only detected vp1 immunopositive cells in multiple islets of 61% (44 of 72)

recent onset type 1 diabetics, compared with only 6% (3 of 50) normal neonatal and pediatric controls, but they found that vp1 staining was restricted only to beta cells. These investigators also showed that in the islets that were vp1 positive, 87% were also positive for the dsRNA-activated protein kinase R (PKR), a protein upregulated by cells during viral infection to establish an antiviral state. The positivity for PKR in the vp1 positive beta cells indicated persistent viral infection of these cells, according to the investigators.

Without fulfilling the requirements of Koch's postulate, a firm causal relationship between any infectious agent and T1D will be difficult to establish. Nevertheless, mounting evidence now favors a role for enteroviral agents in T1D. The risk of highly prevalent viral infections (such as enteroviruses) in predisposing susceptible individuals to T1D, however, compounds the difficulty of predicting who in the general population will actually develop the disease.

Biomarkers in Clinical Use for T1D

The most commonly utilized predictive markers for disease include HLA typing and serum autoantibodies. As mentioned previously, autoantibodies have been shown to appear in the blood of diabetics decades prior to disease onset [1]. These autoantibodies can recognize protein tyrosine phosphatase IA2, islet cell antigen 512 (together called ICA), insulin (also known as IAA), glutamic acid decarboxylase (GAD), and zinc transporter ZnT8. In clinical practice and prospective research studies, the combination of HLA genotype assessment and autoantibody positivity is used to predict T1D development among relatives of T1D probands. The DAISY study (Diabetes Autoimmunity Study of the Young), for example, designed to examine autoimmunity in first degree relatives of T1D patients who

have a high risk HLA genotype, determined that the risk of diabetes in relatives of T1D probands with positivity for two or more of these autoantibodies (ICA, IAA and GAD) was 39% within three years, rising to 68% within 5 years [27, 28].

While T1D prediction in relatives of probands is achievable with the use of these current assessment tools, their predictive value in the general population is far inferior. High risk HLA-DQ and DR genotypes occur in only 2% of the population, of which only 30-40% of HLA-DQ and DR positive patients develop T1D [4]. Furthermore, in the prospective follow-up that included children in the general population, investigators of the DAISY study have found only 8.2% of children to have at least one positive autoantibody [29]. The false positive rate in this cohort was 31%, of whom none developed T1D during follow-up. The transient expression of some of these autoantibodies in some subjects, combined with lack of methodological standardization of the antibody assays among test centers contributed to the false positivity rate [29]. Steps have been subsequently taken to standardize autoantibody assays. However, the transient nature of some autoantibodies has forced investigators to continue their search for better, permanent biomarkers predictive of disease, while turning to a combination of tools for risk factor prediction. For example, in the TrialNet natural history study (NHS) of T1D among relatives of type 1 diabetics, investigators are using HLA typing, serial determination of autoantibodies to glutamic acid decarboxylase (GAD), protein tyrosine phosphatase IA2 and insulin, and serial assessments of response to oral glucose tolerance tests to predict the risk for T1D development [30]. This trial is additionally assessing new potential biomarkers by testing for blood T cell function, antibody isotype profiles, RNA expression and proteomics.

These studies attest to the yet unmet need for accurate biomarkers that can have wider use in the population. Autoantibodies have also not proven to be specific for virally induced T1D in humans. The most important biomarkers, if discovered, would be used to identify people at risk soon after beta cell destruction commences, for example, as a sequelae of a viral infection, or prior to autoantibody appearance. Reliably identifying patients in the early phases of disease would provide clinicians with a window of opportunity when treatment with therapeutic agents could be more effective in halting disease. Ideally, we would be able to screen non-symptomatic patients at risk for T1D for a range of serum biomarkers, which, based on their profile could identify the exact phase of the disease process in a patient. These markers would ideally be tested using non-invasive procedures, be intransient in their expression, have high predictive value, and be convenient for use in the general population. However, early detection of an autoreactive process would be an unworthy goal if we also did not possess in our arsenal a complement of therapies that could preempt further beta cell destruction, or, better yet, prevent beta cell damage altogether. In an ideal world, treatments would be available for use in every phase of the disease and result in one common outcome – protection of beta cells from autoimmune destruction without damaging other cells of the body.

Current Therapies for T1D

Insulin Replacement Therapy

At the point that patients are definitively diagnosed with T1D (onset of hyperglycemia), a few life-saving options exist for treatment. Since its initial purification and

treatment of diabetic patients in 1922 by Canadian physician Frederick Banting, insulin replacement therapy has been the cornerstone of T1D treatment. Significant advances in the delivery of insulin have been made since it was first injected into a diabetic patient. Formulations ranging from short- to intermediate- to long-acting insulin are available for use via delivery systems that range from subcutaneously injectable syringes to sophisticated pumps that deliver metered doses at the required times. Although an inhaled insulin delivery system was recently brought to market under the trade name Exubera, it was subsequently withdrawn from the U.S. market in 2007 due to lack of consumer demand for the product [31].

Unfortunately, insulin replacement therapy is not curative, and it does not directly address the cause of the disease. Additionally, no insulin regimen can reproduce the precise insulin secretory pattern of the pancreatic islet. As a result, glycemic control is difficult to maintain even among the most disciplined of patients. As mentioned previously, tight glycemic control is necessary for the prevention of long term complications of the disease. T1D patients often succumb to the long term sequelae of diabetic nephropathy, neuropathy or retinopathy, but maintenance of tight glycemic control with intensive insulin therapy has been shown to delay and prevent progression of these complications [32].

Transplantation

In cases where microvascular or macrovascular disease has compromised organ function, as in end-stage renal disease, dual pancreas-kidney transplant is considered standard of care [31]. Solitary pancreatic grafts are performed primarily in diabetic patients

with frequent, severe hypoglycemia or extreme blood glucose lability under an optimized diabetes management regime [33]. Islet transplantation, a highly specialized procedure involving implantation of donor islets into a diabetic recipient's liver via the portal vein, is considered a less invasive method for beta cell replacement than traditional solid organ transplantation [34-36]. However, like solid organ transplantation, islet transplantation requires the lifelong use of immunosuppressive therapy to prevent graft rejection [33].

The development and refinement of the Edmonton Protocol, guidelines for islet cell transplantation, however, has allowed for the use of cyclosporin- and steroid-free immunosuppressive regimens in recent years [33]. These regimens are designed to decrease the systemic side effects of earlier steroid-based immunosuppression and their various deleterious side effects. Even with steroid free immunosuppressive therapy, however, fewer than 10% of islet transplanted patients experience insulin independence for 5 years [35]. C-peptide levels, on the other hand, were maintained in 80% of patients up to 5 years post islet transplantation, and the hypoglycemic score, lability index and HbA_{1c} levels were significantly better than at baseline in those that retained detectable C-peptide levels [35]. Another multi-center trial of islet transplantation using the Edmonton protocol recently indicated that only 44% of transplant recipients were able to maintain insulin independence and adequate glycemic control 1 year after transplantation [37]. Only 31% of all transplanted patients remained insulin independent at 2 years, in this same study. Some of the identified barriers to successful islet transplantation have included (1) technical barriers in islet isolation and transplantation, including selection of implantation site (2) recurrent autoimmunity and alloimmune rejection and (3) metabolic dysfunction in islet transplantation

recipients [38]. The need to overcome these current challenges is being met by research aimed at optimizing islet transplantation protocols, understanding the nature of the immune attack against endogenous and transplanted islets, and finding new, unique or combination therapies that can abate recurrent autoimmunity, alloimmunity and metabolic dysfunction.

Islet Pathology in T1D

An understanding of the extent of beta cell destruction in the islets of patients before they become diabetic, at the time of diabetes onset, and years following disease onset might allow us to more rationally design therapies that address the various stages of disease. Results from the Diabetes Prevention Trial (DPT-1) and other studies have depicted the progressive loss of insulin secretion from pre-diabetes to post diagnosis [39]. In some studies, investigators have reported that in human T1D the degree of islet inflammation is mild, with only a small proportion of islets affected by the autoimmunity when compared with animals models of the disease [40]. Pipeleers and colleagues report that only 3-4% of all islets in prediabetic patients are affected by insulitis, and this percentage increases only moderately at the time of diabetes diagnosis [41]. Other reports claim that by the time a patient presents with clinical symptoms of T1D, only 10-20% of their beta cell mass may be remaining [3]. There is no doubt that the degree of remaining beta cell mass is subject to significant inter-individual heterogeneity. It becomes obvious when one considers the age at onset of disease, degree of metabolic decompensation at diagnosis, and environmental determinants dictating the intensity of the beta cell destructive process, that the residual beta cell mass in new onset diabetics may span a wide range. However, at the time of diabetes

onset this beta cell mass is sufficiently diminished to precipitate clinical symptoms of hyperglycemia [3].

In investigations of islets from patients with long standing T1D, investigators have shown that beta cells, although severely reduced in number, are present in the pancreata of T1D patients, even up to 20 years following disease onset [42]. These investigators demonstrated persisting residual insulin positivity in about 40% of patients with T1D after disease duration of greater than 11 years, data that has since been corroborated by other investigators [43]. Variations in the rate of progression of beta cell loss among type 1 diabetics may be due to the waxing and waning of the inflammatory response in response to new antigens, metabolic demands, or other factors [39, 44]. This inflammation has been demonstrated to stimulate beta cell regeneration, but at the same time drive a widening autoimmune response [44]. The persistence of residual beta cell mass, albeit insufficient to control hyperglycemia, has led investigators to hypothesize that residual islets capable of surviving the immune mediated destruction, might have regenerative potential, resist further immune onslaught, and, serve as targets of therapies aimed at improving residual beta cell function.

Emerging Therapies for T1D

Ideally, beta-cell cytoprotective therapies with minimal side effects could be administered to patients before clinical onset as preventive therapies. Given that highly sensitive and specific markers of disease prediction or intervention are currently unavailable, we cannot easily predict who, among those at risk, will actually develop the disease, and

derive any benefit from a given intervention [33]. There are a number of preventive trials testing antigen specific and antigen non-specific approaches to intervene before the autoreactive process begins [33] (**Table 1 and Table 2**). The success of some of these agents is still pending analysis, but several have already shown no effect on disease prevention.

Table 1. Completed, Ongoing, and Planned Prevention Trials in T1D Using Antigen-Specific Approaches

Agent	Route	Stage of development	Details
Insulin	Parenteral	Pilot, completed 1993	Small, pilot study, suggestive of efficacy
Insulin	Parenteral	Pilot, completed 1998	Small, pilot study, suggestive of efficacy
Insulin (DPT-1)	Parenteral	Large efficacy study, completed 2002	No effect seen on disease progression
Insulin (DPT-1)	Oral	Large efficacy study, completed 2005	No effect seen on disease progression; however, strong evidence from subanalysis of significant treatment effect on subjects with strong evidence of insulin autoimmunity. Repeat study planned.
Insulin (INIT 1)	Intranasal	Phase I, completed 2004	No acceleration of loss of beta cell function in individuals at risk for T1D. Immune changes consistent with mucosal tolerance to insulin detected
Insulin (DIPP)	Intranasal	Phase I (ongoing)	
Insulin (INIT II)	Intranasal	Phase II, started December 2006	Randomized, double blind, placebo-controlled trial of intranasal insulin (1.6mg or 16mg)
Insulin	Oral	Efficacy study, planned	Repeat of oral arm of DPT-1
Insulin	Oral and intranasal	Pilot study, planned	Pre-POINT study; dose finding in children with high genetic risk for T1D

Table 2. Completed, Ongoing and Planned Prevention Trials in T1D Using Non-Antigen-Specific Approaches

Agent	Route	Stage of development	Details
Ketotifen (histamine antagonist)	Oral	Pilot, completed 1994	No effect
Cyclosporin	Oral	Pilot, completed 1996	Delay but not prevention in high-risk group
Nicotinamide (Deutsche Nicotinamide Intervention Study, DENIS)	Oral	Efficacy study, completed 1998	No effect
Nicotinamide - European Nicotinamide Diabetes Intervention Trial (ENDIT)	Oral	Efficacy study, completed 2004	No effect
Various nicotinamide combinations (plus cyclosporin, intensive insulin therapy, vitamin E)	Oral	Pilots 1994-2005	No additive effects
Bacille Calmette-Guerin (BCG)	i.d.	Various pilot studies	No effect
Dietary gluten elimination	Oral	Pilot, completed 2002	No effect on autoantibodies or disease
Vitamin D3	Oral	Phase I, ongoing	
Hydrolysed cow's milk (TRIGR)	Oral	Phase I, ongoing	
Docosahexaenoic acid (DHA)	Oral	Pilot, ongoing	

i.d. = intradermal

Since new onset diabetics have already been detected to have the disease, and may still be amenable to disease modulation, it seems more rational to perform intervention trials in patients that have been newly diagnosed with T1D. The goal of new therapies in these patients is to preserve/restore beta cell mass and ablate pathogenic reactivity to autoantigens while preserving the body's normal immune system [33]. The preservation of beta cell mass is now believed to significantly impact the long-term disease outcome by improving glycemic control [33]. The Diabetes Control and Complications Trial (DCCT) demonstrated that patients with residual beta cell function and glycemic control, who have stimulated C-peptide levels >0.2 pmol/ml, have improved responses to treatment and outcomes [33]. The downside to interventions in new onset diabetics is that beta cell damage may already be extensive, limiting the efficacy of new treatments in arresting the autoimmune process. Regardless, several strategies are currently being employed for both prevention and reversal of T1D in humans. Most of these agents are primarily immunomodulating agents, and fall under the broad categories of antigen specific and antigen non-specific agents [33] (**Table 3 and Table 4**). Of these, anti-CD3, an antigen non-specific agent, has proven to be the most efficacious. Anti-CD3 therapy in new onset diabetics has been demonstrated to have multiple effects. It is purported to modulate the TCR-CD3 complex thereby rendering cells 'blind' to antigen and/or induce apoptosis of activated autoreactive cells [45]. Importantly, in human trials, it has been demonstrated to preserve beta cells by halting C-peptide decline and additionally promote Treg production [46]. This antibody is currently being tested in combination therapy with other preventive, immunomodulatory or beta cell regenerative agents to increase long term tolerance to self antigens. Despite the wide array of single

regimen and combination regimen therapies in clinical trials, none has yet been added to current treatment protocols for new onset disease. Research investigating novel therapeutic targets marches on in rodent models. The work presented in this thesis aspires to add a novel candidate, leptin, to the list of emerging therapies. In this thesis, we will demonstrate that leptin's unique duality of function in both protecting the beta cell and restraining the autoimmune response make it a superior candidate to existing monotherapies in trials today.

Table 3. Completed, Ongoing, and Planned Intervention Trials in T1D Using Antigen-Specific Approaches

Agent	Route	Stage of development	Details
NBI-6024 (APL of insulin)	s.c.	Phase I, completed	
Insulin B chain plus incomplete Freund's adjuvant	s.c.	Phase I, completed	
DiaPep277 (hsp60 peptide)	s.c.	Phase II, completed	Phase II in adults reports preservation of C-peptide at 12-18 months
Proinsulin peptide vaccine	i.d.	Phase I (ongoing)	
GAD65	s.c.	Phase I, completed in LADA	Phase II completed in children with T1D, report awaited
Proinsulin-based DNA vaccine (BHT-3021)	i.m.	Phase I planned	

s.c. = subcutaneous; i.d = intradermal; i.m. = intramuscular

Table 4. Completed, Ongoing and Planned Intervention Trials in T1D Using Non-Antigen Specific Approaches

Agent	Stage of development	Details
Cyclosporine	Various trials completed 1984-96	Remission induced in recent onset patients, therapy suspended due to unacceptable side effects
Nicotinamide	Pilot	No effect
Anti-thymocyte globulin plus prednisolone	Pilot	Reduced insulin requirements **>** 100 days after therapy; severe transient thrombocytopenia
Bacille Calmette-Guerin (BCG)	Pilot	No effect
Diazoxide	No effect	
IFN-γ	Phase I	Small pilot, possible effect
PRODIAB (oral protease)	Phase I	No effect
Anti-CD3 MoAb hOKT3g1 (Ala-Ala)	PhaseI/II completed 2002	Remission out to 18 months
Anti-CD3 MoAb ChAglyCD3 (TRX4)	Phase II completed 2005	Reduced insulin requirement out to 18 months
hOKT3g1 (Ala-Ala)	Phase II	Ongoing. Two courses of the Ab administered one year apart.
hOKT3gl (Ala-Ala)	Phase II	Ongoing. Single course administered **to** patients with T1D of 4-12 months duration since diagnosis
hOKT3g1 (Ala-Ala)	Phase II/III	Planned
ChAglyCD3 (TRX4)	Phase II/III	Planned
anti-CD20 MoAb (Rituximab)	Phase II	Ongoing
Anti-thymocyte globulin (ATG)	Phase I	Ongoing
Anti-thymocyte globulin (ATG)	Phase II	Planned
Anti-CD52 (Campath-1H)	Phase I	Planned
Autologous umbilical cord blood cells	Phase I	Ongoing
Autologous gene-engineered DCs	Phase I	Starts 2007

Rodent Models of T1D

A variety of rodent models have enabled us to dissect the pathogenic mechanisms of T1D, identify new targets, and test new therapies. But, no one rodent completely mimics the natural history of human disease. There are a variety of spontaneous and inducible models in both mice and rats, the most widely used of which is the non-obese diabetic (NOD) mouse. Even though the NOD mouse has served as the cornerstone of research in T1D, it actually does not share many features in common with human T1D. Furthermore, given the increasing recognition of the importance of viruses in human disease, it seems rational to utilize a robust model of virally induced T1D to develop insights into mechanisms of disease and to test therapies that can intervene with this form of disease.

The genetically susceptible Biobreeding Diabetes Resistant (BBDR) rat is a superior model to the NOD in addressing virally mediated disease. The treatment of BBDR rats with a combination of a TLR 3 ligand, polyinosinic:polycytidilic acid (pIC) and a virus, Kilham rat virus (KRV) has been shown to precipitate diabetes in 100% of rats, whereas many experimental viral infections in NOD mice actually prevent or delay T1D [47]. Several other attributes of the pIC + KRV model in the BBDR rat make it appealing for research studies. First, there is no gender bias for T1D in these rats, as in humans. NOD mice, in contrast, have a predilection for T1D in females more than males. Second, pIC + KRV treated rats develop life-threatening DKA, as do humans. Third, the degree of cellularity and composition of immune infiltrates of islets in these rats resemble that of human diabetic islets [47]. Fourth, thyroiditis, an autoimmune disease that can develop in humans with T1D, can

also be induced concomitantly with diabetes in BBDR rats by treating them with a regulatory T cell depleting antibody (anti-RT6.1) in conjunction with pIC [48].

Viral Induction of T1D in BBDR Rats

BBDR rats harbor the genetically susceptible $RT1^u$ MHC haplotype, but do not develop T1D in a viral antibody free environment. Natural infection with KRV induces diabetes in 1% of animals while deliberate inoculation of KRV via intraperitoneal (i.p.) injection induces diabetes in 25-40% of rats within 2-4 weeks [49]. Studies in BBDR rats have demonstrated that infection with other viruses such as sialodacroadenitis virus and vaccinia virus does not induce diabetes [50, 51]. Therefore, disease precipitation is virus specific in this model. Precipitation of T1D by KRV is also age-dependent, with weanling rats (21-25 days old) developing disease, and older rats remaining resistant to disease induction [47].

KRV is one of three ssDNA parvoviruses of rodents that have been identified. H-1 and rat parvovirus-1 are the others in the family. KRV has three overlapping structural proteins, VP1, VP2 and VP3, and two overlapping nonstructural proteins, NS1 and NS2. The VP1 and VP2 proteins of KRV and H-1 are 77% homologous while their NS1 and NS2 proteins are 100% homologous [52]. H-1, however, does not cause diabetes in rodents. This attribute is beneficial in utilizing H-1 as an ideal control in experiments using KRV. The inability of H-1 to cause diabetes despite sharing a high degree of sequence homology with KRV can be explained by the differences in the tissue tropisms of these viruses. KRV has been documented to infect primarily dividing T and B cells, endothelial cells and

megakaryocytes [53, 54]. H-1, in contrast, predominantly infects nonlymphopoietic organs such as the kidney [55].

The mechanism of KRV induced, autoimmune diabetes is still only partially understood. The fact that it directly infects immune cells responsible for beta cell destruction is germane to its mechanism of disease induction. Certain mechanisms of disease precipitation in this model have been ruled out. These include direct beta cell cytolysis and molecular mimicry (defined as a shared epitope between pancreatic beta cell proteins and KRV specific peptide). Since KRV was found not to directly infect beta cells, investigators concluded that it did not contribute directly to beta cell cytolysis [53]. Molecular mimicry was ruled out as a possible mechanism contributing to autoimmunity because infection with recombinant vaccinia virus (rVV) expressing KRV peptides VP1, VP2, NS1 and NS2, did not result in autoimmune diabetes. Investigators found that even though each of the KRV viral peptide was clearly expressed following rVV infection, and viral peptide specific T cells and antibodies against KRV peptides were generated, none of the rats developed insulitis or diabetes [56].

One mechanism of immune dysregulation that has been proposed in this model, for which evidence is now mounting, includes a shift in the balance between regulatory T cells (Tregs) and effector T cells (Teff) toward autoreactive cells [52, 56, 57]. Chung et al. [56] were the first to demonstrate that seven days following infection with KRV, there is an upregulation of cytotoxic CD8+ T cells and Th1-like CD45RC+CD4+ T cells (high IFN-γ mRNA expression) and downregulation of CD45RC-CD4+ Th2-like T cells (high IL-4 and TGF-ß mRNA expression) in the spleens of infected animals compared with uninfected

controls. BBDR rats had been previously shown to harbor thymic epithelial defects resulting in the generation of thymocytes that can escape negative selection and circulate as autoreactive cells in the periphery. The presence and destructive potential of these autoreactive cells were unmasked when they induced diabetes following adoptive transfer into athymic recipients [58]. As a result, Chung and colleagues hypothesized that BBDRs may have autoreactive T cells in their periphery, which are normally kept in check by regulatory cells. In the presence of KRV infection, the subsequent polarization toward a Th-1 response expands these cells to become autoreactive effector T cells that are beta cell destructive [56].

Additional studies have supported the hypothesis of immune imbalance contributing to KRV induced T1D. These studies have further attributed the imbalance to strong activation of the innate immune response by KRV. In 2003, Zipris and colleagues reported that KRV infection downmodulated regulatory T cells in the spleen while increasing effector CD8 populations [52]. In 2005, the same investigators showed that pretreatment with a variety of either purified TLR agonists or heat killed bacteria synergized with KRV infection to increase the penetrance of diabetes in BBDR rats [50](**Table 5**). The cumulative incidence of diabetes in BBDR rats ranged between 50-100% and depended upon which TLR agonist was injected. Furthermore, pretreatment with TLR agonists lowered the threshold dose of KRV that could precipitate diabetes in BBDR rats. Zipris and colleagues concluded from this study that the addition of TLR agonists to the KRV model system enhanced the ability of diabetogenic infectious agents to induce disease in a genetically susceptible host.

Table 5. Frequency and Latency to Onset of Diabetes in BBDR Rats Following Administration of Virus and Various TLR Ligands

Group	Virus	TLR ligand	TLR	Dose (µg/g Body Weight)	Frequency of Diabetes (%)	Days
1	KRV	None			13/57 (23)	19 (16-40)
2	KRV	Poly (I:C)	3	1	14/14 (100)	14 (11-14)
3	None	Poly (I:C)		1	0/15 (0)	
4	KRV	LPS	4	2	14/18 (78)	17 (13-25)
5	None	LPS		2	0/5 (0)	
6	KRV	Zymosan	2,6	2	8/15 (53)	17 (16-25)
7	KRV	PGN	2,6	5	5/8 (63)	14 (14-20)
8	KRV	R848	7,8	2	4/8 (50)	17 (16-18)
9	KRV	CpG	9	2	10/11 (91)	13 (13-16)

Table 5. BBDR rats 22-25 days of age of either sex were randomized as they became available to nine treatment groups. Rats in group 1 were injected with 1 X 10^7 PFU of KRV. Rats in groups 3 and 5 received a 3-day course of the indicated TLR ligands and no further treatment. Rats in all remaining groups were injected on 3 consecutive days with the indicated TLR ligands and on the following day injected i.p. with 1 X 10^7 PFU of KRV. All animals were tested for the onset of diabetes for 40 days after the final treatment. Diabetes was defined as the presence of a plasma glucose concentration >250mg/dl (11.1 mM/L) on 2 consecutive days. Susceptibility to diabetes induction among rats treated with the combination of a TLR ligand plus KRV (groups 2,4, and 6-9) was significantly greater than in rats treated with KRV alone (group 1, $p < 0.05$ in all cases).

In 2007, Zipris and colleagues further elucidated the mechanism by which KRV promotes a pro-inflammatory environment that precipitates autoimmunity [49]. Based on *in vitro* experiments, they demonstrated that KRV infected BBDR splenocytes increased production of proinflammatory cytokines such as IL-6 and IL-12p40. The investigators showed that virus infection was not required for this increase in IL-12p40, rather, genomic DNA isolated from KRV infected rat kidney cells could also induce IL-12p40 from splenocytes. On the basis of this result, the investigators hypothesized that the ligand inducing splenic secretion of IL-12p40 might be viral ssDNA acting on endosomal TLR9 receptors. To test this hypothesis, Zipris and colleagues cultured BBDR splenocytes in the presence of KRV, CpG DNA, a synthetic TLR9 ligand, or pIC in the presence or absence of chloroquine, a known inhibitor of endosomal acidification. Addition of chloroquine to BBDR spleen cells in the presence of either KRV or CpG resulted in significant reduction in the secretion of IL-12p40 in a dose dependent manner. These results, nevertheless, prompted the investigators to hypothesize that chloroquine might attenuate KRV-induced autoimmune diabetes in BBDR rats. They treated 22-25 day old BBDR rats with pIC + KRV to induce diabetes, then randomized them to receive a daily injection of either saline or chloroquine (100 µg/g body weight) for 21 days. Only 40% of chloroquine treated rats became diabetic following pIC and KRV, compared with 88% of saline treated controls. The investigators concluded that chloroquine may attenuate KRV induced diabetes in the BBDR rat by decreasing activation of the innate immune response.

In light of their previous research into pIC + KRV induced diabetes in the BBDR rat, Zipris and colleagues have proposed the following explanation for the mechanism of T1D in

this model. They propose that pretreatment of BBDR rats with pIC followed by infection with KRV activates APCs, promotes their maturation, which then enhances the presentation of islet autoantigens to the immune system. This presentation facilitates the generation of autoreactive CD4+ and cytotoxic CD8+ T cells that can migrate to the islets and cause insulitis, beta cell destruction, and diabetes.

Metabolic Hypothesis of Autoimmune Diabetes

While significant insights into the possible mechanisms underlying virally induced T1D have been gleaned from the pIC + KRV model, most of the research in this model, as well as other autoimmune models of T1D, has focused solely on the dysregulation of the immune system. We hypothesize that in addition to immune imbalance, metabolic dysregulation may be responsible for pIC + KRV induced diabetes. One way of testing the role of metabolic dysregulation in precipitating T1D would be to test therapies that can correct the predominant metabolic abnormalities observed in this disease, namely hyperglycemia and insulin loss. Recent advances in type 2 diabetes research have elucidated the role of various adipokines in regulating insulin secretion from beta cells. The identification and functional evaluation of these adipokines has prompted us to scrutinize the effects of one adipokine in particular, leptin, in our virally induced model of T1D.

Leptin is 16kDa protein hormone, secreted primarily by adipocytes, that plays a key role in regulating a wide range of biological responses including energy homeostasis, neuroendocrine function, lymphopoiesis, angiogenesis, bone formation and reproduction

[59]. The dual role of leptin in regulating the immune and endocrine systems, in particular, has made it an attractive target of further research in both type 2 and T1D in recent years.

Leptin Deficiency in ob/ob and db/db Mice

With regard to its endocrine effects, the most obvious evidence of the importance of leptin in energy balance and glucose homeostasis comes from *ob/ob* and *db/db* mice. These mice suffer from a syndrome of obesity, diabetes, infertility, hypothyroidism, hypercorticoidism, low sympathetic activity, and impaired thermoregulation. Interestingly, both mice are also immunodeficient, with reduced splenic and thymic weight, and lymphopenia. Parabiosis studies between *ob/ob* and lean mice revealed that the genetic mutation resulted in the deficiency of a lipostatic factor [60], which Zhang and colleagues later identified as leptin, the product of the *ob/ob* gene, attributing the obesity phenotype to its deficiency [61]. Subsequent studies revealed that mutation in the long isoform of the leptin receptor, Ob-Rb, was the genetic mutation responsible for the obesity observed in *db/db* mice [62]. Peripheral administration of leptin to *ob/ob* mice suppressed food intake in these mice, corrected infertility, reversed hyperglycemia, hypertriglyceridemia and hyperinsulinemia, and increased their body temperature and metabolic rate [63-66].

Effect of Leptin on Beta Cell Function

The effect of overexpression of leptin in rodent models has demonstrated some important and beneficial effects of this hormone on metabolism. In 1996, studies on normal male wistar furth (WF) rats revealed that overexpression of leptin using adenoviral vectors (AdLeptin) was associated with reduced food intake, reduced body weight gain, compared

with normoleptinemic controls, and complete disappearance of visible body fat in hyperleptinemic rats, when compared with pair fed controls [67]. Serum triglyceride (TG) and free fatty acid (FFA) levels were reduced in hyperleptinemic rats and these rats exhibited lower plasma insulin levels than pair fed animals or controls treated with adenovirus bearing an irrelevant gene construct, beta galactosidase (AdBetagal). In this study, investigators demonstrated that despite the decline in plasma insulin levels in hyperleptinemic rats, blood glucose levels remained normal. They concluded that even with a 60% reduction in plasma insulin levels, maintenance of normal blood glucose levels was evidence of increased insulin sensitivity by peripheral tissues [67].

Subsequent studies have shown that exogenous leptin treatment can decrease insulin secretion from beta cells. Long term treatment of beta cells with leptin was demonstrated to decrease insulin biosynthesis [68] and secretion [68-70]. The mechanism by which leptin has been demonstrated to inhibit insulin secretion is by activating the ATP-sensitive K+ channel in beta cells and thereby hyperpolarize the beta cells to resist depolarization required for insulin secretion [69, 71, 72]. In 2001, Okuya and colleagues showed that addition of 1 and 5nM recombinant leptin to rat islets cultured for 24 hours reduced their TG content and suppressed iNOS mRNA expression [73]. Furthermore these concentrations increased viable beta cell number, which these investigators attributed to the antiapoptotic effects of leptin mediated at least in part by the reduction in endogenous TG. Leptin, therefore, is hypothesized to promote beta cell survival by inhibiting insulin secretion, decreasing metabolic stress and depleting intracellular, lipotoxic TG.

Role of Leptin in Immunity

In addition to its metabolic effects on beta cells, leptin's role in lymphopoiesis and the innate and adaptive immune systems has also received increased scrutiny in recent years. Due to its structural similarity to other long chain helical cytokines [74] such as IL-6, IL-12, IL-15, granulocyte-colony stimulating factor (G-CSF), leptin has been increasingly recognized as a cytokine-like hormone with pleotropic actions on the immune system. The thymic atrophy and reduced lymphocyte function in *ob/ob* and *db/db* mice attests to the importance of leptin in hematopoiesis. Suppressed T cell activation in *ob/ob* mice has correlated with protection from experimentally induced autoimmune diseases in these mice, including experimental allergic encephalomyelitis (EAE) and antigen induced arthritis [75-78].

Early studies demonstrated that proinflammatory cytokines such as TNF-α and IL-1 and IL-6 and TLR ligands such as LPS increased serum leptin levels, which was believed to contribute to the anorexigenic effects of these cytokines during inflammatory responses [79]. Subsequent studies have revealed that leptin itself promotes the secretion of these proinflammatory cytokines by selectively promoting the proliferative and cytokine secretory function of naïve T cells *in vitro*. Leptin induced T cell expansion and cytokine secretion has been demonstrated to reverse starvation induced immunosuppression in mice [80]. Additional confirmation of leptin's role as a growth factor for T cells comes from leptin repletion studies in *ob/ob* mice. Exogenous administration of murine leptin in *ob/ob* mice also protects these mice from starvation induced lymphoid atrophy by increasing thymic CD4+CD8+ populations [81]. Leptin has effects on other immune cell types as well. It has

been demonstrated to activate monocytes, dendritic cells (DCs) and macrophages, *in vitro,* and stimulate them to produce Th1 type cytokines [82]. However, it was recently shown to be a negative regulator of T regulatory (Treg) cell proliferation. This effect of leptin was elegantly demonstrated using an anti-antibody which, by blocking the effects of leptin on its receptor, promoted Treg proliferation *in vitro* in the presence of anti-CD3, anti-CD28, and IL-2 stimulation [83]. Overall, leptin treatment has been demonstrated to generate a pro-inflammatory immune response in rodent models, an attribute that may not appear to be beneficial in autoimmune prone models.

Association of Leptin with Autoimmune Diseases

Increased leptin levels have been associated with several autoimmune diseases of humans and rodents. Leptin treatment of *ob/ob* mice restores their susceptibility to EAE [84]. Increased leptin levels in EAE mice have been associated with reduced frequency of CD4+CD25+ Tregs [85]. These data have been supported in humans with multiple sclerosis, among whom increases in leptin also correlated with reduced numbers of Tregs [85]. Investigators have additionally found a significant increase in serum leptin levels in patients with acute ulcerative colitis (UC), compared with controls, a finding precipitated by the observation that acute stages of UC are characterized by anorexia and weight loss [86].

Role of Leptin in Type 1 Diabetes

Several lines of research are now pointing to the proinflammatory role of leptin in precipitating T1D. In 2002, Matarese and colleagues observed that female NOD mice aged 4-26 wks had a statistically significant increase in serum leptin compared with male NODs and

other mouse strains (SJL, BALB/c, C57BL/6J) [87]. They observed a surge of serum leptin in these mice during the weeks immediately preceding the onset of hyperglycemia, with levels rising 5- to 10 times above normal. Interestingly, there was no significant association between this surge and changes in the animal's body weight or food intake. Leptin's reported anorexigenic properties leading to lack of weight gain, therefore, did not seem to apply with the level of serum surge in this case. Following the surge in serum leptin in these NOD mice, there was an observed drop that accompanied establishment of clinical diabetes.

The investigators then tested a protocol for exogenous leptin administration in newborn NOD mice to determine if generating a serum surge in younger mice might accelerate disease. Newborn NOD mice treated with 1µg/g body weight of mouse recombinant leptin i.p. weekly until 4 weeks, then every 36 hrs for 2 consecutive weeks, experienced rapid acceleration of diabetes. By the sixth week following the initiation of treatment, 90% of leptin treated female mice developed diabetes. Among PBS treated mice, 85% became diabetic, but only by 6 months. There was a higher severity of disease in leptin treated female mice and rapid evolution to DKA, with one third of treated mice dying from ketoacidosis within 2-3 days of the onset of hyperglycemia. Rapid evolution to DKA is a relatively rare phenomenon in NOD mice, underscoring the importance of these findings. The investigators found that mRNA expression in the splenic periarteriolar sheaths of leptin treated mice was rich in IFN-γ mRNA expressing cells. They claimed that their results in total were indicative of leptin promoting proinflammatory cell responses in NOD mice and directly influencing the development of autoimmune T1D mediated by Th1 responses.

Complementing this sentinel study, investigators at Jackson Laboratories discovered a leptin resistant NOD/LtJ-Lep$^{db\text{-}5J}$ mouse that had a mutation in the extracellular domain of the leptin receptor, Ob-Rb [88]. This mouse developed a metabolic syndrome characterized by juvenile obesity, hyperglycemia, hyperinsulinemia, and hyperleptinemia, but failed to develop T1D. The mice were found to have fewer splenic beta cell autoreactive CD8+ T cells (NRP-V7+) than wild type NODs [89]. Additionally, in a series of elegant adoptive transfer studies, the investigators demonstrated that cells from the NOD/LtJ-Lep$^{db\text{-}5J}$ could not transfer disease to a variety of immune deficient recipients [89]. The investigators concluded that the mechanism of beta cell protection in the NOD/LtJ-Lep$^{db\text{-}5J}$ mice was the inhibition of activation of T effector cells in the absence of appropriate signaling of leptin via its receptor.

A newer study in 2007 investigated the role of prenatal undernutrition in predisposing newborn NOD mice to T1D. Oge and colleagues determined that a 40% restriction of energy intake applied to pregnant NOD dams from day 12.5 to 18.5 of gestation led to undernourished/low birth weight female offspring that had a 35% decrease in the cumulative incidence of T1D [90]. Maternal leptin levels were significantly lower in the diet restricted dams at the end of the diet restriction period compared with dams fed *ad libitum.* Low maternal leptin levels corresponded with significantly higher leptin levels in the undernourished offspring, but these offspring were more protected from T1D than the normal birth weight controls. Although the two studies cannot be directly compared due to the difference in body weight of the newborn NOD mice, the Matarese study in 2002 and this study both achieved a hyperleptinemic state in newborn NOD mice, but observed different

outcomes with T1D incidence. Whether leptin can have a protective effect against T1D *in utero* compared with post partum remains to be determined.

In 2008, Unger and colleagues elucidated a new role for leptin in T1D. They tested the hypothesis that leptin could replace the therapeutic actions of insulin in different rodent models of beta cell destruction, one of which included the NOD mouse [91]. They treated new onset NOD diabetics with AdLeptin or AdBeta-gal and demonstrated that AdLeptin treatment of NOD diabetics normalized blood glucose and corrected ketosis in mice that were hyperglycemic prior to treatment. Although the normoglycemia was transient (10-80 days), mice that reverted to normoglycemia had no measurable plasma insulin or detectable pancreatic preproinsulin mRNA. Hyperleptinemia (319 ± 76 ng/ml) in this instance decreased food intake in NOD mice to 51% of the control diabetic NODs, but the weight loss in these mice halted after AdLeptin treatment, compared with AdBetagal treated NODs who remained hyperglycemic and continued to lose weight following adenovirus treatment. Thirty days post treatment, plasma glucagon levels were significantly higher in AdBetagal diabetics than AdLeptin treated diabetics. The investigators concluded that leptin-mediated suppression of diabetic hyperglucagonemia may have contributed to the reversal of the diabetic state in these mice [91]. In light of these new findings, we deduce that despite the reported pro-inflammatory and pro- autoimmune effects of leptin, its metabolic effects on beta cells and other glucose regulatory cells necessitates its further evaluation as a therapeutic agent in T1D models.

Summary of Aims

Autoimmune T1D predominantly affects young children, and imposes a significant burden on their lives stemming from its chronicity and its potentially life-threatening complication, DKA. To decrease the burden of this disease, we would ideally detect it early, and provide treatments that could altogether prevent disease onset, or at a minimum, prevent its complications. No reliable biomarkers currently exist to detect the disease in the general population. There are some predictive biomarkers in clinical use, primarily, HLA typing and autoantibodies, that can predict the risk for family members of probands. However, these markers are not reliable for use in the general population. Part of the reason for the paucity in biomarkers for this disease is due to the heterogeneity in its causal factors and mechanisms of disease precipitation. Viruses have long been hypothesized to be precipitating agents of T1D in genetically susceptible individuals. The effects of viruses in triggering T1D may explain the heterogenous nature of disease onset and disease kinetics in the general population. Furthermore, treatments that are targeted toward virally mediated disease may decrease disease incidence and improve disease prognosis. We can enhance our understanding of the mechanisms of virally mediated disease in humans with the use of rodent models.

In this study, we will use the BBDR rat model of virally mediated T1D to achieve the following aims: (1) identify predict biomarkers of virally mediated T1D in genetically susceptible rats and test the potential of a therapeutic agent, leptin, in (2) preventing disease, (3) reversing new onset disease and (4) preventing autoimmune rejection of transplanted islets. We will use global proteomics techniques to mine the sera of virally induced diabetic rats to identify proteins that may serve as biomarkers in human disease. We will validate

these biomarkers in various other virally mediated rat models of T1D to test their predictive value. We will test the effects of an adipokine, leptin, as a therapeutic agent in virally mediated T1D in BBDR rats to determine if it has protective effects on beta cells. Previous studies have shown that a hyperleptinemic state can protect beta cells from apoptosis and prevent insulin secretion. We hypothesize that the beta cell cytoprotective effect created by a hyperleptinemic state may prevent the autoimmune destruction of beta cells in the pIC + KRV induced diabetes. Furthermore, we will test the hypotheses that the metabolic effects of a hyperleptinemic state can stabilize new onset disease and perhaps even protect islets transplanted into diabetic rats.

CHAPTER II. MATERIALS AND METHODS

Animals

Viral-Ab-free BBDR/Wor and BBZDR/Wor rats of either sex were obtained from BRM, Inc. (Worcester, MA). Animals were certified to be free of Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, rat corona virus, Kilham rat virus (KRV), H-1, GD7, Reo-3, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, mouse adenovirus, Hantaan virus, and Encephalitozoon cuniculi. All animals were housed in a viral-Ab-free facility under standard laboratory conditions, provided with water and commercial chow *ad libitum*, until used. All animals were maintained in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996) and the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. BBDR rats 22–25 days of age of either sex were used in all experiments involving virus induction of T1D. Thirty to 40 day old female BBDR/Wor rats were used for all *in vitro* lymphocyte stimulation and proliferation assays described below.

Viruses

KRV. KRV UMass isolate and the normal rat kidney (NRK) cell lines were obtained from stocks maintained in our laboratories. NRK cells were cultured in high-glucose complete Dulbecco's Modified Eagle Medium (cDMEM) containing 10% heat-inactivated FBS, 1mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50µM 2-mercaptoethanol (Invitrogen Life Technologies, Carlsbad, CA). KRV at a multiplicity of

infection of one was used to infect NRK cells; virus was harvested from supernatant of infected cultures two days after infection and kept at –70°C until use. Viruses were titered using a plaque assay as previously described [52].

Adenovirus. AdCMV-leptin (AdLeptin) is a replication-defective recombinant adenovirus that expresses the rat leptin gene under the control of the cytomegalovirus (CMV) promoter. AdBetagal is a recombinant adenovirus expressing the ß-galactosidase gene under control of the CMV promoter. Seed stocks of these viruses were generously provided by Dr. Roger Unger (University of Texas Southwestern Medical Center, Dallas, TX). Adenoviruses were propagated in L293A cells. Viruses were prepared by infection of L293A cell monolayers and purified on $CsCl_2$ gradients [92]. Adenovirus titers were determined based on the infection of L293A cells with serial dilutions of virus stock. The virus titer was determined by quantitation of hexon protein-positive L293A cells by immunohistochemistry at the appropriate serial dilution of the stock virus.

H-1 virus and RCMV were the generous gifts of Dr. Danny Zipris (Barbara Davis Center for Childhood Diabetes, University of Colorado, Denver, CO) and Dr. John Mordes (University of Massachusetts Medical School, Worcester, MA), respectively.

Reagents

Polyinosinic:polycytidylic acid (pIC) was purchased from Sigma (St. Louis, MO), dissolved in Dulbecco's PBS (1 mg/ml), sterile filtered, and stored at -20ºC until used. The concentration of contaminating endotoxin was determined commercially (Charles River Endosafe, Charleston, SC) and was uniformly <10 units/mg. Lyophilized rat recombinant

leptin (rrLeptin) was purchased from R&D Systems (Minneapolis, MN) and stored at -20^{o}C until reconstituted. Lyophylized protein was reconstituted with sterile 20mM Tris HCl, pH 8 to a concentration of 2.5 mg/ml, stored at 4^{o}C, and used within 2 weeks of reconstitution.

Antibodies

Purified, biotinylated or fluorochrome-conjugated anti-rat mAbs to CD3 (G4.18), TCR (R73), CD4 (OX-35 or W3/25), CD8 (OX-8), CD28 (JJ319), CD25 (OX-39), CD11b/c (OX-42), CD45RA (B cell marker) (OX-33), RT1B (class II MHC marker) (OX-6), NKRP1A (clone 10/78), CD86 (24F), mAbs and isotype controls were obtained from BD Biosciences (San Jose, CA). Fluorochrome-conjugated anti-rat/mouse Foxp3 antibody (FJK-16a) was obtained from eBiosciences (San Diego, CA).

Leptin and Insulin RIA

Leptin RIA assays were performed by Dr. Young Lee (University of Texas Southwestern Medical Center, Dallas, TX). Insulin RIA assays were performed and results reported by Millipore (Billerica, MA).

ELISA Assays

Haptoglobin ELISA kit was purchased from Immunology Consultants Lab (Newberg, OR) and used according to the manufacturer's instructions. A standard curve was used with the haptoglobin ELISA kits, utilizing standards supplied in the kit. All test samples were quantified in duplicate, and any values out of range were appropriately diluted and reanalyzed.

Blood Collection/Serum Harvesting

In all experiments involving long term follow up of rats, 200 - 500µl blood was collected on indicated days via tail vein nicking into unheparinized tubes. On experiment termination days, rats were first sacrificed in a CO_2 chamber and blood was subsequently harvested by cardiac puncture. All blood samples were allowed to clot at room temperature for 1 hr, spun at 4^{o}C at 12000 RPM in a microcentrifuge for 10 minutes, and serum harvested. Serum used for proteomics experiments (see below) were additionally respun at 4^{o}C at 12000 RPM in a microcentrifuge for 10 minutes to remove any residual cell debris. All sera were aliquoted and frozen at -80^{o}C until further use.

Serum Proteomics Methods

Immunoaffinity Removal of Rat Serum Abundant Proteins

Rat serum samples were depleted of albumin, IgG and transferrin using the Multi Affinity Removal Column (Ms-3, 4.6x100mm, Agilient Technologies). Sera were first diluted with 4 volumes of Agilent solvent A and centrifuged at 16000 x g for 1 minute through a 0.22 µm spin filter to remove particulates. Five hundred microliters of the diluted serum (1:10) was then injected onto an Applied Biosystems Vision™ Workstation liquid chromatography system with the attached Agilent Multi Affinity Removal Column at a flow rate of 0.5 ml/min. Flow through was collected in a volume of 1.5 mls, and an aliquot was analyzed by one-dimensional (1D) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins captured by the column were removed with Agilent solvent B at a flow rate

of 1 ml/min and analyzed in parallel with the flow-through samples. The column was regenerated by equilibrating with solvent A.

Native and Non-native One-Dimensional Electrophoresis

Nondepleted serum samples were quantified using the Bradford dye-binding assay, and 3µg of total protein from each sample was loaded onto a 8-16% tris-HCl precast gels (Biorad). Gels were run in tris glycine buffer without sodium dodecyl sulfate or any denaturing agents at a constant voltage of 200V for 50 minutes. Depleted samples were concentrated on Amicon Ultra 4 10kDa cutoff ultracentrifugation columns (Millipore). Protein quantitation of the concentrated sample was achieved using the Bradford dye-binding assay. Four micrograms of the concentrated samples were reduced with 50mM DTT and subjected to electrophoresis on 4–12% Bis-Tris SDS NuPage gels (Invitrogen) according to the manufacturer's protocol. Gels were run with the MOPS SDS running buffer (Invitrogen) at a constant voltage of 200V for 50 minutes. Both native and non-native one dimensional gels were stained overnight with coomasie blue and destained with milliQ water prior to imaging.

Two-Dimensional Electrophoresis

Prior to isoelectric focusing (IEF), samples were solubilized in 40mM Tris, 7M urea, 2M thiourea and 2% CHAPS, reduced with tri-butylphosphine, and alkylated with 10mM acrylamide for 90 minutes at room temperature. Protein was precipitated from each sample by mixing it with nine times the sample volume of acetone for 30 minutes at room temperature. Following precipitation, the pellet was again solubilized and buffer exchanged

in 7M urea, 2M thiourea, and 2% CHAPS until conductivity was < 150 µS/cm, and 100µg protein were subjected to IEF on 11cm pH 4–7 and pH 6-11 immobilized pH gradient (IPG) strips (Amersham). Following IEF, IPG strips were equilibrated in 6M urea, 2% SDS, 50mM Tris-acetate buffer (pH 7.0), and 0.01% bromophenol blue and subjected to SDS-PAGE on 8–16% Tris-HCL Gel (Bio-Rad, Hercules, CA). All gels were stained in Sypro® Ruby (Molecular Probes, Eugene, OR) and imaged by a charge-coupled device camera on a fluorescent imager (Bio-Rad Gel-Doc).

Axima QIT MALDI MS Analysis

Gel spots excised with an automatic gel cutter (Bio-Rad) were first washed in 1mL of water to remove any residual acid, then in 1mL of a 50% ACN: 50% 50mM ammonium bicarbonate solution for 30 min. ACN (200µl) was added to shrink the gel, the excess removed, and the gel dried using a SpeedVac. Digestion was performed by the addition of 50-100 ng trypsin (Sigma) in 20µl volume of 25mM ammonium bicarbonate with incubation at 37^{o} C overnight. The supernatant was then decanted, gel slices soaked in 50µl of 80% ACN and 1% formic acid to shrink the slices, dried using a SpeedVac to a 10µl volume, and acidified with 1-2µl of 1% TFA. Samples were loaded on a µC18 Zip Tip (Millipore) pre-equilibrated with 0.1% TFA. After washing with 2 x 10µl aliquots of 0.1% TFA, samples were deposited directly onto the MALDI sample target using 1µl of Matrix solution (15 mg/ml of 2,5 Dihydroxybenzoic acid) (MassPrep DHB, Waters Corp., Mildford, MA) in 50:50 ACN : 0.1% TFA. Samples were allowed to air dry prior to insertion into the mass spectrometer. Analysis was performed on a Kratos Axima QIT (Shimadzu Instruments)

matrix-assisted-laser desorption/ionization (MALDI) mass spectrometer. Peptides were analyzed in positive ion mode in mid mass range (700-3000 Da). The instrument was externally calibrated with P14R (1533.86 Da) and ACTH (18-39) (2465.20 Da). Precursors were selected based on signal intensity at a mass resolution width of 250 for CID fragmentation using Argon as the collision gas. Database searches were performed with Mascot (Matrix Sciences, Ltd.) using the rat IPI database and Peptide Mass Fingerprint program for MS data and the MS/MS Ion Search program for CID data. Since low abundant samples typically provide an insufficient number of peptides to make an identification based solely on MS information, all identifications were confirmed or established with CID (MS/MS) data.

iTRAQ Labeling

iTRAQ analysis was based on a single experiment. Sera from the first day following KRV infection was used from one rat in each of three treatment groups: PBS alone, pIC alone or pIC + KRV. Sera from each treatment group were depleted of abundant proteins as described above, and an equal volume of each of the three sera was used to create a pooled control group. Four total depleted samples (three from each of the treatment groups and one the pooled control) were labeled with iTRAQ reagents, according to the manufacturer's instructions. Each such sample (100µg) was precipitated with 10 volumes of chilled acetone by incubating in -20°C for 1 hour, centrifuged, washed with 1 ml of 20% acetonitrile and vacuum dried. 0.5M Triethyammonium bicarbonate and 2% sodium dodecyl sulfate (SDS) were added to dissolve the protein pellet before mixing with 50mM Tris-(2-carboxyethyl) phosphine (TCEP) as a reducing agent. The reducing samples were incubated at 60°C for 1

hour and then cysteine-blocking reagent (200mM methyl methanethiosulfonate, MMTS) was added at room temperature for 10 min. Each sample was then digested by treating with 10µg of trypsin (2 µg/µl) in the presence of $CaCl_2$ overnight at 37 °C, vacuum dried and then redissolved in 30µl of 0.5M Triethyammonium bicarbonate. For each digested sample, 70 µl of iTRAQ reagent (isobaric reagents 114, 115, 116 and 117, Applied Biosystem Inc., prepared according to manufacturer's instructions) was added to each sample and incubated at room temperature for 1 hour. Following derivatization, the 4 samples (one reference sample labeled with the 114 reagent, and three individual samples labeled with the other three reagents) were mixed and vacuum dried, then resuspended in 3 ml of Strong Cation Exchange (SCX) loading buffer (25% v/v acetonitrile (ACN), 10 mM KH_2PO_4, pH 3, with phosphoric acid).

Sample Fractionation

The combined peptide mixture was separated by strong cation exchange (SCX) chromatography column (Polysulfoethyl column, 4.6 mmID x 50 mm, 5u, 200A; The Nest Group Inc). The column was first equilibrated with SCX loading buffer. The pH of the iTRAQ sample was verified to be <3.3. Additional SCX loading buffer was added if necessary to adjust pH. The sample was then loaded at the rate of approximately 1 drop/second onto the SCX column and washed with 1 ml of SCX loading buffer to remove excess reagent. Peptides were eluted with a linear gradient of 0–1000 mM KCl (in 25% v/v ACN, 10 mM KH_2PO_4, pH 3). Five fractions of the elutant were collected for analysis with LC-MS/MS.

LC-MS/MS Analysis of iTRAQ Labeled Samples

Each fraction was dissolved in 70µl of 2% acetonitrile (ACN), 0.1% Triflouroacetic acid (TFA) in water and 10µl was injected using the CapLC Autosampler (Waters Inc.) The peptides are first trapped on a Peptide CapTrap column (Michrom Bioresources Inc.) at a flow rate of 12 µl/min of 2% ACN, 0.1% Formic acid (FA), for 12 min. The flow direction through the trapping column is then reversed and reduced to 250 nl/min and directed through the analytical capillary column. The analytical column is a 75 µm ID X 10 cm PicoFrit column packed with ProteoPep™ II C18, 300 Å, 5 µm (NewObjective). A 90 min solvent gradient from 2% B to 17% B in 6min, 17%-45% in 84 min followed by 10 min at 90% B was passed through the trapping column and analytical column (solvent A = 2% ACN, 0.1% FA, solvent B = 90% ACN, 0.1% FA).

Data was acquired on a Q-Tof Premier mass spectrometer (Waters Inc) using data dependent acquisition in which the instrument cycled through acquisitions of a full-scan TOF mass spectrum, following which, three MS/MS spectra were recorded sequentially on the three most abundant ions present in the full scan. The three most abundant, multiple charged peptides (2+ to 4+) above 10 count threshold in the MS scan with m/z between 400 Da and 2000 Da were selected for MS/MS. Dynamic exclusion was set for each selected peak at ±1.8 Da for 90 seconds. A lock mass spectra of glufibrinogen were collected every 30 seconds.

iTRAQ Data Analysis

The raw data was processed using PLGS (Waters, Inc.), the data was lock-mass corrected, deisotoped, charge state reduced, and a .pkl file was generated for each fraction. The .pkl

files were combined and searched with Mascot™ using the rat IPI database(EBI) version 3.38, and a decoy database was searched for false positive detection and determined to be <1%. Mascot search parameters used included: peptide mass tolerance set at 100ppm, fragment tolerance at 0.1Da, full trypsin specificity, variable modifications of M+16, C+MMTS, and fixed modifications of Kn+iTRAQ. Mascot also performed the iTRAQ ratio calculations.

Western Blot Analysis of Serum Haptologbin

The Bradford assay was used to quantify protein levels from rat sera. Three micrograms of each serum sample were reduced by BME, loaded onto 4-20% precast tris-glycine gradient gels (Invitrogen) along with purified haptoglobin (Life Sciences) and ladder marker (Gibco), and run at 150V for 1 hour. Gels were transferred onto PVDF membranes, blocked with 5% milk for at least 1 hour, then placed in primary antibody (chicken anti-rat haptoglobin, Abcam) for 2 hours. Membranes were washed three times with TBS+tween, then placed in secondary antibody (rabbit anti-chicken HRP, Abcam) for 1 hour. Membranes were washed two times with TBS+tween then once with TBS alone and developed with ECL (Gibco). Membranes were imaged using Kodak chemiluminescent film.

Histology

All pancreas specimens that were obtained from rats in indicated studies were fixed in 10% buffered formalin, and stained with hematoxylin and eosin. Pancreas sections were additionally stained for the presence of insulin and glucagon. All pancreas specimens were evaluated for inflammatory cells, insulin and glucagon. Insulitis was scored as follows: 0, no

infiltration; 1+, peri-islet infiltration only; 2+, infiltration of some but not all islets; 3+ infiltration of most islets; 4+, end-stage islets.

Preparation of Spleen and Lymph Node Single Cell Suspensions

Rats were killed in an atmosphere of 100% CO_2, and cervical and pancreatic lymph nodes and spleen were removed, as indicated in the experiment, and processed aseptically. Single cell suspensions of lymph nodes and spleens were obtained by gentle extrusion between frosted glass slides and 50 mesh wire screens, respectively, into medium (RPMI 1640). Cells were filtered through a 70µm filter, spun at 4°C at 1500 RPM for 5 minutes, decanted and resuspended in RPMI medium. An aliquot of the single cell suspension was counted with the Beckman Coulter Z2 cell counter (Fullerton, CA).

CFSE Labeling

Single cell suspensions of lymph node pools and spleens ($50x10^6$ cells of each tissue) were washed with RPMI, spun and resuspended in 1µM CFSE in 37°C PBS. CFSE labeled cells were incubated in a 37°C water bath for 15 minutes. Following the incubation, cells were washed three times in PBS with 3% FBS, counted with the Beckman Coulter Z2 cell counter and resuspended to the appropriate concentration for plating in T cell culture media. This media was comprised of RPMI medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS (Hyclone, Logan, UT, USA), 1% Pen/Strep/Glut (Gibco, Carlsbad, CA, USA), and 0.5% β-mercaptoethanol (Gibco).

In vitro T Cell Stimulation

Unlabeled or CFSE labeled lymph node cells and splenocytes were plated into either 6 or 12 well plate (BD Falcon, Bedford, MA, USA) at a concentration of 2×10^6 cells per well in 3 ml of T cell culture media. Cells were either unstimulated or stimulated with 0.1 μg/ml, 0.5 μg/ml or 1 μg/ml of anti-CD3 monoclonal antibody in a volume of 3 mls of RPMI medium per well at 37°C. Where indicated, soluble anti-CD28 mAb (2.5 μg/ml, clone JJ319; BD Pharmingen) was also added.

Flow Cytometry

BBDR CLNs, PLNs, spleens were harvested and prepared into single-cell suspension by mechanical disruption, washed two times in RPMI and counted with a Beckman Coulter Z2 cell counter. One to two million cells from single-cell suspensions of indicated tissues were washed two times with serum-free, $Ca^{2+}Mg^{2+}$-free Dulbecco's phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA). Cells were then stained at 4^oC for 30 min with LIVE/DEAD blue (Molecular Probes) to visualize and exclude dead cells. Cells were then washed twice with PBS, containing 1.0% fetal clone serum, 0.1% sodium azide, before incubating them with the appropriate fluorescent antibodies against cell surface markers. For analyses involving intracellular Foxp3 antibody staining, cells that were surface stained were further incubated with Cytofix/Cytoperm for 20 minutes at 4^oC to permeabilize cell membranes. Samples were washed once with Cytowash and incubated with the APC-conjugated anti-rat/mouse Foxp3 antibody for 20 minutes at 4^oC. Following this incubation, samples were washed twice with Cytowash and resuspended in PBS containing 2%

paraformaldehyde (Poly-Sciences, Warrington, PA). Labeled cells were analyzed with a FACSCalibur or LSRII instrument (BD Biosciences). For *ex vivo* cell analysis, at least 100,000 events were collected, unless specified elsewhere. FlowJo software (Tree Star, Ashland, OR) was used to analyze flow data after acquisition.

Islet Isolation Procedure

Ninety to 120 day old BBDR donor rats were anesthetized and pancreas ballooned, by injection through the bile duct, with 10-12 mls of a 1.0 mg/ml collagenase P (Roche) solution diluted with HBSS. Pancreata were washed with warm RPMI and incubated at 37^{o}C for 25 minutes to allow for complete collagenase digestion of the tissue. Digestion was halted with cold RPMI with 1% horse serum. Pancreata were allowed to settle to bottom of the tube, the supernatant was decanted, and the tissue was then washed with cold RPMI with 1% horse serum. The tissue was then mechanically disrupted with a 14 gauge needle and a 10cc syringe. Disrupted tissue was spun at 1000rpm for 10 seconds, following which the supernatant was decanted. The procedure for mechanical disruption was repeated once more, following which the tissue was spun, resuspended in a 1% RPMI solution, strained through a metal sieve and rinsed with 1% RPMI. Disrupted tissue was spun at 1200 rpm for 10 seconds. Supernatants were aspirated, and pancreatic tissue was respun to remove as much of the supernatant as possible. Pellets were resuspended in 10ml of Histopaque 1077 and gently homogenized by tapping, then overlaid with 10ml of 1% RPMI. This gradient was spun at 1800 rpm for 20 min at 20^{o}C without brakes. Islets were collected from the interface of the Histopaque and RPMI and placed into a tube with RPMI with 5% horse serum. Islets were then washed twice with 50ml of 5% RPMI and spun at 1200 rpm for 10 seconds at room

temperature. Islets were then placed in a 100mm petri dish in 10mls of 5% RPMI under a dissection microscope and handpicked for counting. Ten islets per gram body weight of transplant recipient were aliquoted and mixed with 15 ml RPMI (no serum) and spun at 1200 rpm for 30 seconds. The supernatant was aspirated and the wash was repeated once more. Following the final wash, islets were placed in a 250µl eppendorf tube and allowed to settle to the bottom prior to transplantation.

Alzet Pump Priming and Surgical Insertion

Alzet pump models 2001 and 2ML2 were purchased from the Durect Corporation (Cupertino, CA). Each 2001 model pump holds a volume of 200µl of the test solution, and is designed to deliver 1µl of the test solution per hour per day for up to 7 days. The 2ML2 model pump holds 2 mls of volume, and is designed to deliver 5µl of the test solution per hour per day for up to 14 days. Both alzet pumps models were loaded with either a 2.5 mg/ml solution of rrLeptin or vehicle (20mM Tris HCl, pH 8). Based on the concentration of rrleptin loaded into each pump, we determined that the 2001 model pump would deliver 60µg of rrLeptin per pump per day, and the 2ML2 pump would deliver 300µg of rrLeptin per pump per day.

In all diabetes protection studies, two model 2001 alzet pumps were used per rat. For diabetes reveral studies and islet transplantation studies, one model 2ML2 alzet pump was used per rat. Model 2001 pumps were not primed prior to insertion. Model 2ML2 pumps were primed with 0.9% sterile saline in a 50 ml falcon tube placed in a 37^0C water bath

overnight to allow for immediate release of the test solution upon implantation in the rats that were already diabetic.

Anesthesia Protocol

During all surgical procedures (pump insertion, pump replacements and uninephrectomies) rats were anesthetized with ketamine/xylazine administered i.p. at a dose of 87/13 mg/kg body weight. Buprenex was administered i.p. at a dose of 0.02-0.08 mg/kg body weight for analgesia. Isofluorane was administered via nose cone on an as needed basis during the surgery. Prior to the surgery, the dorsum of rats was shaved and skin surface sterilized with a 1% topical solution of betadine followed by alcohol swab. This sterilization process was repeated three times. A small incision (<5mm) was made on the dorsal flank and either two model 2001 pumps or a single model 2ML2 pump was inserted into the subcutaneous space created by a hemostat. Following pump implantation the skin incision was closed with autoclips. Yohimbine was injected i.p. at a dose of 0.2 mg/kg body weight to reverse the effects of xylazine. Surgical autoclips were removed either during a pump replacement procedure or no later than 10 days following their last placement.

For rats undergoing islet transplantation followed by pump insertion, a small incision (<5mm) was first made on the left dorsal flank that penetrated into the abdominal cavity. The left kidney was then held in place as syngeneic islets (8-12 islets/g body weight) were inserted under its capsule. The incision through the abdominal wall was sutured using vicryl coated suture (Ethicon, Sommerville, NJ). A subcutaneous space was then created on the same flank by a hemostat for the implantation of the 2ML2 pump. Following pump

implantation, the skin incision was closed with autoclips. Yohimbine was injected i.p. at a dose of 0.2 mg/kg body weight to reverse the effects of xylazine.

Surgical Procedure for Uninephrectomy

A small incision (<5mm) was made on the left dorsal flank that penetrated into the abdominal cavity. The islet bearing kidney was isolated and clamped with a hemostat at the closest entry point of the renal vasculature into the hilus. The renal vasculature was then ligated twice with duct suture, followed by unclamping of the hemostat, and dissection of the islet bearing kidney. Rats were observed briefly to ensure that the vasculature was appropriately ligated and that there was no hemorrhage into the abdominal cavity. The incision through the abdominal wall was sutured using vicryl coated suture, and the skin incision was closed with autoclips. Yohimbine was injected i.p. at a dose of 0.2 mg/kg body weight to reverse the effects of xylazine.

Experimental Protocols

pIC+KRV Diabetes Induction Protocol

Male and female BBDR/Wor rats were injected intraperitoneally (i.p.) with pIC (1-2 µg/g body weight) on three consecutive days starting at 21-25 days of age. These treatment time points will be referred to as day -3, -2, and -1. On the fourth day (day 0), rats received a single i.p. dose of 1 x 10^7 PFUs of KRV, H-1 or RCMV, as indicated in experiments. Rats were tested for glycosuria (test strips, Clinistix, Bayer, Elkhart, IN) twice weekly starting at 7 days post virus infection. Diabetes was confirmed by documenting two consecutive plasma

glucose concentrations >250 mg/dL (Accu-Chek Aviva, Roche Diagnostics, Indianapolis, IN).

AdLeptin Diabetes Protection Protocol

Male and female BBDR rats, aged 22-24 days, were injected i.v. with $1x10^{10}$ infectious particles of AdLeptin or AdBetagal per 0.5 ml PBS per 100g body weight on the day before the start of the pIC + KRV induction protocol (day -4). pIC was subsequently administered i.p. at a dose of 1-2 µg/g body weight for three consecutive days (days -3, -2, -1). On the fourth day, in the indicated experiments, rats received a single i.p. dose of $1x10^7$ pfu of KRV (day 0). Diabetes onset was confirmed as described above. Blood was harvested for serum collection by tail nicking rats on the following days: day -4 (prior to adenovirus injection), day 0 (prior to KRV injection), day +14, and day +45 post KRV. As rats became diabetic, on the second day of high blood glucose, a single insulin pellet was implanted subcutaneously to restore normoglycemia. On day +45 post KRV, all non-diabetic rats and diabetic rats treated with insulin pellets were sacrificed in an atmosphere of 100% CO_2, their blood harvested by cardiac puncture for serum collection, and pancreas harvested in 10% formalin. Paraffin-embedded sections of the pancreas of diabetic and non-diabetic rats were prepared and stained with hematoxylin and eosin (H&E); additional sections were stained for the presence of insulin and glucagon by immunohistochemistry (IHC). Serum samples from this study were analyzed for rat leptin and insulin levels. Rat serum leptin levels were detected by radioimmunoassay (courtesy of Dr. Young Lee). Rat serum insulin levels were also detected by radioimmunoassay (Millipore).

rrLeptin and Diabetes Protection Protocol

Alzet pumps (model 2001) were loaded with either a vehicle solution or a 2.5 mg/ml solution of rrLeptin. On the first day of the virus induction protocol, six hours prior to pIC treatment, two alzet pumps were inserted into the lateral dorsum of BBDR/Wor rats employing the surgical procedures detailed previously. Rats were injected i.p. with pIC for three consecutive days, followed by KRV on the fourth day. Diabetes onset was confirmed as described above. Alzet pumps were replaced every 5 days following the first installation, for up to 6 times total. These pump replacement days corresponded with day +1, +6, +11, +16, +21 post KRV treatment. On day 26 post KRV, the pumps were removed from all rats. In certain experiments, as indicated, rats were sacrificed on day +6, prior to the second pump replacement. Their CLN, PLN and spleens were harvested, and single cell suspensions of each organ was generated for flow cytometry analysis as described in further detail below. Body weights were recorded at least three times weekly from the time of pump installation until animals were sacrificed. Rats were tail bled twice weekly by tail vein nicking for serum analysis of various hormone levels including rrLeptin. Diabetic rats were sacrificed on the second day of high blood glucose or at the end of the study period (day +45 post KRV). Tissues were harvested at the time of sacrifice as described in the AdLeptin diabetes protection study. Rat serum leptin levels were detected by radioimmunoassay (courtesy of Dr. Young Lee).

rrLeptin and Diabetes Reversal Protocol

BBDR rats induced to become diabetic with either 1µg/g or 2 µg/g body weight of pIC, as indicated in the relevant experiment, followed by a single dose of KRV. Seven days after

they received KRV i.p., these rats were tested for glycosuria daily to determine the exact day of diabetes onset. Blood glucose levels were documented on the first day of diabetes onset. On the second consecutive day of high blood glucose, rats were tail bled for serum harvest, then implanted with one 2ML2 alzet pump loaded with either rrLeptin (2.5 mg/ml) or vehicle control on the left dorsal flank. Diabetic rats in this protocol were not treated with an insulin pellet upon disease onset. Pump implanted rats were carefully monitored daily for their state of activity, blood glucose levels and ketonuria (urine test strips, Ketostix, Bayer, Elkhart, IN). Any rat that appeared moribund or exhibited ketonuria with a blood glucose level >250 mg/dl was euthanized. Blood was harvested by cardiac puncture for serum and pancreas was fixed in 10% formalin for IHC.

AdLeptin and Islet Transplantation Protocol

BBDR rats induced to become diabetic as described above were implanted subcutaneously with insulin pellets (Linplant, LinShin, Toronto, Canada) for up to 7-10 days following the second high blood glucose (>250 mg/dl). Four to five days following the insertion of the insulin pellet, male and female rats were injected i.v. with a single dose of $1x10^{10}$ infectious particles of AdLeptin or AdBetagal per 0.5 ml PBS per 100g body weight. Insulin pellets were retained in adenovirus treated rats for a few days following the i.v. injection to allow for maximal adenoviral gene expression. Seven to ten days after the insulin pellets were first inserted (three to six days after i.v. adenovirus injection), the pellets were removed and syngeneic islets were transplanted under the kidney capsule at a concentration of 8–12 islets/g body weight.

rrLeptin and Islet Transplantation Protocol

BBDR rats induced to become diabetic as described above were implanted subcutaneously with insulin pellets for up to 7-10 days following the second high blood glucose. At the end of this time period, insulin pellets were removed, and syngeneic islets were transplanted under the kidney capsule at a concentration of 8–12 islets/g body weight. One 2ML2 alzet pump containing either rrLeptin or vehicle control was inserted into the subcutaneous space of the same flank as the islet-bearing kidney, as described in the surgical methods section. Pumps were replaced ten days following the first insertion, and removed entirely ten days following replacement.

Monitoring Diabetes Recurrence in Islet Transplanted Rats

In both the AdLeptin and the rrLeptin islet transplantation protocols, rats were monitored for graft rejection by detection of blood glucose three times weekly following transplantation. Graft failure was defined as the first day on which plasma glucose concentration exceeded 250 mg/dl. Diabetic rats were sacrificed, cardiac blood harvested for serum, and pancreas and islet transplanted kidney fixed in 10% formalin for IHC. In all rats that remained non-diabetic at the end of the study period, we established the presence of a functional graft and lack of residual endogenous islet function by performing a unilateral nephrectomy of the islet bearing kidney, and documenting the recurrence of hyperglycemia for two consecutive days following the uninephrectomy. Rats that became diabetic at this point were sacrificed, cardiac blood harvested for serum, and pancreas and islet transplanted kidney fixed in 10% formalin. Paraffin-embedded sections of pancreas and graft-bearing kidneys of diabetic and

non-diabetic rats were prepared and stained with H&E; additional sections were stained for the presence of insulin and glucagon by IHC.

Statistical Analyses

Average duration of diabetes free survival is presented as the median. Diabetes-free survival among groups was compared using Kaplan and Meier survival analysis. The equality of graft survival or diabetes-free distributions for animals in different treatment groups was tested using the log rank statistic. Comparisons of means of two groups used the student's T test and comparisons of three means or more used one-way analyses of variance (ANOVA) with the Bonferroni test for a posteriori contrasts (GraphPad Prism 4.0 Software, San Diego, CA).

CHAPTER III. PROTEOMIC PROFILING OF SERUM FROM pIC + KRV TREATED RATS IDENTIFIES HAPTOGLOBIN AS A POTENTIAL BIOMARKER FOR TYPE 1 DIABETES

Introduction

Due to the availability of new therapies such as anti-CD3, which may preserve beta cells if administered early in the course of T1D, there is a high demand in the scientific community for noninvasively testable biomarkers that can identify disease onset before it becomes clinically apparent. The pIC + KRV inducible model of T1D in the BBDR rat resembles human disease more closely than many other spontaneous models of T1D in rodents. Moreover, the binary nature of disease outcome in our experimental system allows us to identify putative serum biomarkers in the earliest phase of the autoimmune process among rats that we know will develop diabetes. Consequently, we hypothesized that a global proteome search could identify protein biomarkers in the sera of pIC + KRV treated rats that appear in the early phases of disease, even prior to the initiation of insulitis and autoimmune destruction of beta cells in the pancreas that herald disease onset.

To capture an early time point for biomarker discovery we chose to analyze sera from blood harvested one day following infection with KRV (day +1). Twenty one to twenty five day old BBDR rats were treated with PBS alone (n=3), pIC alone (1 µg/g body weight; n=3) or pIC (1 µg/g body weight) + KRV (n=3). In this experiment, 100% of the pIC + KRV treated rats developed diabetes within 11-21 days following treatment, compared with 0% of the rats in either the PBS alone or pIC alone treatment groups during the 45 day follow-up

period. We analyzed sera from blood harvested on day +1 from rats in each of the experimental groups. The proteomic workflow described in **Figure 1** outlines the general strategy we used to determine the differences in serum proteomes of rats in each treatment group. By first depleting the sera of the three most highly abundant proteins, albumin, IgG and transferrin, as described in the Materials and Methods chapter, we sought to identify important lower abundance serum proteins that could serve as putative biomarkers of disease induction. We recognized that by depleting sera of albumin, which serves as a carrier molecule for a variety of proteins in the body, we may be excluding a fraction of potential biomarkers that are albumin-bound, although, a strong serum biomarker might still be present for detection in its free, unbound form in the serum.

In this study we report the use of multiple global proteomic profiling techniques to identify early serum biomarkers in the virally inducible model of T1D in BBDR rats. Our screen of the BBDR rat serum proteome has identified several potential biomarkers. The most significant of these is haptoglobin, an acute phase and hemoglobin scavenger protein that appears to have a differential expression pattern in the sera of pIC + KRV treated rats.

Figure 1. Proteomic Work Flow for 2D Gel and iTRAQ Analyses

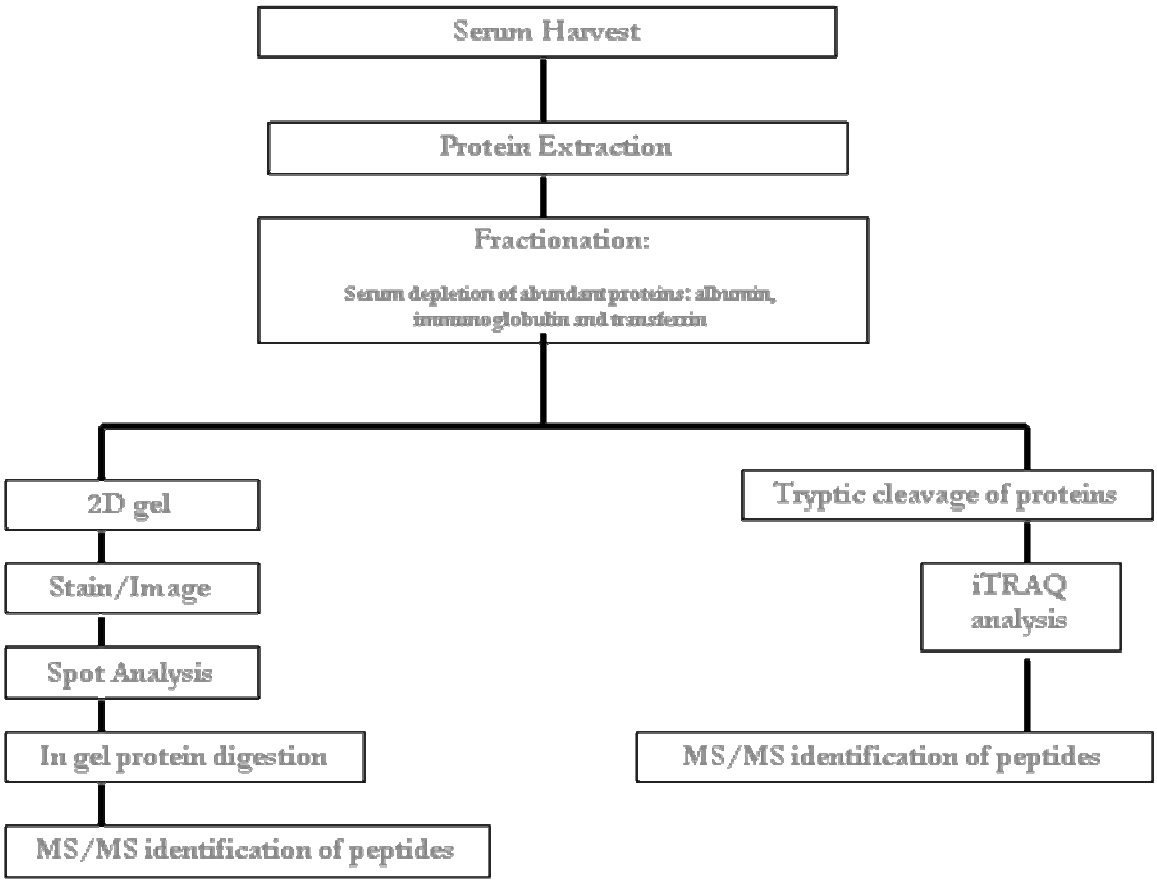

Figure 1. For the 2D gel analysis, three biological replicates/serum samples from rats treated with PBS alone, pIC alone or pIC+KRV on day +1 were run in duplicate for the spot analysis. Differences in spots were based on student T test comparisons between each group. For the iTRAQ analysis, one serum sample from each treatment group, and one pool of serum from each of the three samples was analyzed

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Results

1. 2D Gel Analysis Identification of Haptoglobin as an Early Putative Biomarker for Virally Induced Type 1 Diabetes

The first method we employed for global biomarker screening was 2-D gel electrophoresis followed by spot analysis and protein identification by mass spectrometry. Depleted sera were subjected to 2D gel electrophoresis followed by image analysis of differentially expressed proteins. Proteins whose levels were significantly different on day +1 between the rats in each of the treatment groups were selected for identification by MALDI-TOF-MS/MS (**Table 6**). Of the various proteins identified, most were either acute phase proteins such as α_2 macroglobulin or complement component 3, or proteins involved in hemolysis and the heme scavenging pathway such as hemoglobin, biliverdin reductase B, and hemopexin. The most significant of these differences between treatment groups appeared to be in the protein haptoglobin. Haptoglobin normally circulates in the serum of uninfected rats as an $\alpha_2ß_2$ tetrameric structure ready to sequester free hemoglobin that may be released into the circulation as a result of trauma or inflammation. Such trauma or inflammation can additionally stimulate increased synthesis of activated haptoglobin and its precursor protein, prohaptoglobin, from hepatocytes. Prohaptoglobin is subsequently activated by serum proteases to the active $\alpha_2ß_2$ tetrameric structure [93, 94].

Table 6. Proteins Identified by 2D Gel Electrophoresis and MALDI-MS/MS

Protein ID	Normalized vol	Differential Expression	Measured pI	Measured MW (kDa)	Fold Difference	p value
alpha 1 inhibitor III	0.038	up in PBS only	5.156	38.697	2.324	8.64E-08
alpha 1 inhibitor III	0.051	up in PBS only	4.916	23.87	2.674	0.001
alpha 1 inhibitor III	0.015	up in PBS only	4.497	19.921	2.34	0.008
complement component 3	0.014	up in PBS only	5.834	17.071	2.375	0.046
Spin 2a	0.035	up in PBS only	4.623	17.29	2.437	0.0001016
bal-647	0.046	down in pIC/KRV vs pIC alone	5.388	29.891	-2.245	0.022
bal-647	0.03	down in pIC/KRV vs pIC alone	5.202	30.411	-3.06	0.039
complement component 3	0.019	down in pIC/KRV vs pIC alone	7.921	231.297	-2.115	4.81E-06
haptoglobin precursor	0.052	down in pIC/KRV vs pIC alone	5.36	12.695	-2.624	0.007
haptoglobin precursor	0.068	down in pIC/KRV vs pIC alone	4.994	12.967	-2.854	0.014
haptoglobin precursor or hatoglobin alpha 1s	0.015	down in pIC/KRV vs pIC alone	5.871	30.625	-2.501	0.001
preprohaptoglobin	0.024	down in pIC/KRV vs pIC alone	5.174	32.225	-3.156	0.0001633
preprohaptoglobin	0.009	down in pIC/KRV vs pIC alone	5.902	29.093	-3.579	0.008
alpha 2 macroglobulin	0.098	up in pIC alone and pIC+KRV vs. PBS	5.457	129.799	2.203	0.0002132
alpha 2 macroglobulin	0.1	up in pIC alone and pIC+KRV vs. PBS	5.386	132.628	2.032	0.023
alpha 2 macroglobulin	0.114	up in pIC alone and pIC+KRV vs. PBS	5.42	132.628	2.48	3.04E-05
alpha 2 macroglobulin	0.044	up in pIC alone and pIC+KRV vs. PBS	6.414	42.516	4.059	0.017
alpha 2 macroglobulin	0.197	up in pIC alone and pIC+KRV vs. PBS	6.319	30.937	3.258	0.007
alpha 2 macroglobulin	0.112	up in pIC alone and pIC+KRV vs. PBS	6.723	30.564	2.693	2.59E-05
alpha 2 macroglobulin	0.049	up in pIC alone and pIC+KRV vs. PBS	6.309	32.089	4.336	0.003
alpha and beta globin	***0.085***	***up in pIC alone and pIC+KRV vs. PBS***	***7.7***	***12.491***	***6.179***	***0.007***
biliverdin reductase B	0.011	up in pIC alone and pIC+KRV vs. PBS	6.553	23.304	2.97	0.008
complement component 3	0.124	up in pIC alone and pIC+KRV vs. PBS	8.635	61.733	2.841	0.000105
haptoglobin	0.131	up in pIC alone and pIC+KRV vs. PBS	5.23	43.719	3.212	0.0009308
haptoglobin	0.118	up in pIC alone and pIC+KRV vs. PBS	5.114	44.196	2.049	0.002
haptoglobin	0.191	up in pIC alone and pIC+KRV vs. PBS	5.363	42.562	2.488	0.0007297
haptoglobin	***0.086***	***up in pIC alone and pIC+KRV vs. PBS***	***5.694***	***41.24***	***9.642***	***0.0003603***
hemopexin	0.016	up in pIC alone and pIC+KRV vs. PBS	6.685	50.229	2.112	6.86E-05
hemopexin	0.029	up in pIC alone and pIC+KRV vs. PBS	6.685	46.493	2.016	0.0001485
major beta hemoglobin	0.078	up in pIC alone and pIC+KRV vs. PBS	7.312	13.037	2.904	4.92E-07
major beta hemoglobin	0.027	up in pIC alone and pIC+KRV vs. PBS	7.004	12.199	2.822	0.011
plasminogen protein	0.054	up in pIC alone and pIC+KRV vs. PBS	7.095	166.928	2.399	0.001

Table 6. List of differentially expressed proteins identified in the sera of rats treated with PBS alone (n=3), pIC alone (1µg/g body weight; n=3) or pIC (1µg/g body weight) + KRV (n=3) on day + 1 of follow-up. Sera were depleted of the three most abundant proteins: albumin, transferrin and immunoglobulin, subjected to isoelectric focusing followed by 2D gel electrophoresis, image analysis, gel spot excision and protein identification by MALDI-TOF-MS/MS. Only spots that were significantly different between any two groups were excised for protein identification. Student's T test was used to compare differences in the average normalized volume of gel spots between treatment groups. A $p<0.05$ was considered statistically different.

We observed that several modified variants of the activated haptoglobin protein were identified by MALDI-TOF-MS/MS (**Table 6**). These isoforms ranged in pI from 5.1 to 5.6 and in molecular weight from 41 – 44 kDa. Although we did not identify the modifications that contributed to these isoforms, all isoforms appeared to be significantly elevated (between two and nine fold higher) in the sera of rats treated with pIC alone or pIC +KRV, when compared with rats treated with PBS alone. The mature haptoglobin protein levels did not appear to be different, however, between rats treated with pIC + KRV versus pIC alone. Several haptoglobin precursor fragments, on the other hand, appeared to be significantly lower in rats treated with pIC + KRV than those treated with pIC alone. Interestingly, the other significantly elevated protein (six fold higher) in the sera of pIC alone or pIC + KRV treated rats compared with rats treated with PBS alone included the alpha and beta globin chains of hemoglobin, targets of haptoglobin binding. Because the role of many acute phase proteins in the viral induction of T1D is unknown, the differential expression of haptoglobin as well as other acute phase proteins identified by 2D and MALDI-TOF-MS/MS in pIC + KRV treated rats prompted us to confirm their serum expression using another global proteome profiling technique, iTRAQ (isobaric tag for relative and absolute quantitation).

2. iTRAQ Analysis Confirms Haptoglobin as an Early Putative Biomarker for Virally Induced Type 1 Diabetes

To identify differentially expressed proteins in sera from rats treated with PBS alone, pIC alone or pIC + KRV on day +1 using an alternative proteomic technique, we employed the use of iTRAQ analysis on the same samples used for the 2D analysis. For iTRAQ analysis we pooled an equal volume of sera from each of three rats within a treatment group, giving

rise to one sample per treatment group. One hundred microliters of the pooled sera from each treatment group were then depleted of the three most highly abundant proteins, albumin, immunoglobulin and transferrin, separated into five fractions by strong cationic exchange column, and subjected to iTRAQ analysis. All proteins identified using the iTRAQ are listed in **Table 7.** Of the identified proteins, serum haptoglobin levels appeared to be at least two fold higher in the pIC + KRV treated rats than the pIC alone or the PBS alone treated rats on day +1.

Table 7. Proteins Identified by iTRAQ Analysis and LCMS-MS

pIC alone vs. PBS alone	pIC+KRV vs. PBS alone	pIC+KRV vs. pIC alone	Accession Number	Protein Name
---	1.011	---	IPI00781938	Tax_Id=10116 76 kDa protein
---	**0.569**	---	IPI00215296	Tax_Id=10116 Complement C1q subcomponent subunit A precursor
---	2.295	---	IPI00372792	Tax_Id=10116 Plasma protease C1 inhibitor precursor
0.933	**2.601**	**2.687**	IPI00325610	Tax_Id=10116 Haptoglobin precursor
1.092	2.313	2.04	IPI00476177	Tax_Id=10116 77 kDa protein
1.723	**2.229**	**1.247**	IPI00210900	Tax_Id=10116 AMBP protein precursor
1.365	**1.664**	1.175	IPI00781822	Tax_Id=10116 103 kDa protein
0.573	0.687	1.163	IPI00200591	Tax_Id=10116 Contrapsin-like protease inhibitor 3 precursor
0.754	**0.909**	**1.163**	IPI00210120	Tax_Id=10116 Aa1018
0.578	0.692	1.162	IPI00392216	Tax_Id=10116 Liver regeneration protein lrryan
0.62	0.724	1.125	IPI00200593	Tax_Id=10116 Contrapsin-like protease inhibitor 1 precursor
2.084	2.423	1.121	IPI00195516	Tax_Id=10116 Hemopexin precursor
0.979	**1.048**	**1.113**	IPI00208659	Tax_Id=10116 Complement inhibitory factor H
1.207	1.375	1.098	IPI00324019	Tax_Id=10116 Alpha-1-antiproteinase precursor
1.349	1.514	1.081	IPI00326140	Tax_Id=10116 Alpha-1-macroglobulin
1.118	**1.249**	1.076	IPI00327469	Tax_Id=10116 Alpha-2-HS-glycoprotein precursor
0.872	0.964	1.063	IPI00372372	Tax_Id=10116 Serine (Or cysteine) peptidase inhibitor, clade C (Antithrombin), member 1
1.195	**1.314**	**1.06**	IPI00778633	Tax_Id=10116 Apolipoprotein H
1.193	1.307	**1.056**	IPI00212708	Tax_Id=10116 Fetub protein
2.195	2.386	1.048	IPI00187799	Tax_Id=10116 Isoform HMW of Kininogen-1 precursor
1.325	1.435	1.044	IPI00389806	Tax_Id=10116 LOC498793 protein
0.996	1.074	1.039	IPI00555161	Tax_Id=10116 Aa1064
1.298	1.398	1.037	IPI00194097	Tax_Id=10116 Vitamin D-binding protein precursor
2.083	2.243	1.037	IPI00679245	Tax_Id=10116 T-kininogen 2 precursor
1.944	2.086	1.034	IPI00515829	Tax_Id=10116 Kininogen 1
0.957	1.027	1.034	IPI00188225	Tax_Id=10116 C-reactive protein precursor
1.351	**1.439**	1.026	IPI00422037	Tax_Id=10116 Complement component 4, gene 2
0.73	0.776	1.024	IPI00480639	Tax_Id=10116 Complement C3 precursor
1.276	1.346	1.019	IPI00422011	Tax_Id=10116 B-factor, properdin
0.792	0.837	1.019	IPI00358382	Tax_Id=10116 similar to complement component 8, alpha polypeptide
1.255	**1.326**	**1.018**	IPI00189981	Tax_Id=10116 Prothrombin precursor (Fragment)
1.327	**1.391**	**1.01**	IPI00213036	Tax_Id=10116 Complement C4 precursor
3.507	3.67	1.006	IPI00392886	Tax_Id=10116 Alpha-2-macroglobulin precursor
0.889	0.924	1.002	IPI00200757	Tax_Id=10116 Isoform 1 of Fibronectin precursor
1.171	1.216	1.001	IPI00201262	Tax_Id=10116 Alpha-1-inhibitor 3 precursor
0.603	**0.619**	0.991	IPI00206780	Tax_Id=10116 Plasminogen precursor
1.121	**1.153**	0.991	IPI00198667	Tax_Id=10116 Clusterin precursor
0.677	0.695	0.988	IPI00188338	Tax_Id=10116 similar to Inter-alpha-trypsin inhibitor heavy chain H1 precursor
1.119	1.141	**0.983**	IPI00192302	Tax_Id=10116 99 kDa protein
1.059	**1.074**	0.98	IPI00211075	Tax_Id=10116 Contrapsin-like protease inhibitor 6 precursor
1.234	1.252	0.979	IPI00325847	Tax_Id=10116 GPI-anchored ceruloplasmin
1.316	1.329	0.974	IPI00368704	Tax_Id=10116 similar to Murinoglobulin 1 homolog
1.218	1.221	0.967	IPI00212666	Tax_Id=10116 Rat alpha(1)-inhibitor 3, variant I precursor
1.625	**1.426**	0.967	IPI00207668	Tax_Id=10116 Afamin precursor
0.704	0.703	0.963	IPI00324272	Tax_Id=10116 Apolipoprotein A-IV precursor
1.094	1.072	**0.944**	IPI00563778	Tax_Id=10116 Apolipoprotein A-I
0.902	0.879	**0.94**	IPI00655254	Tax_Id=10116 LRRGT00161
0.731	**0.71**	0.936	IPI00204451	Tax_Id=10116 Complement factor I precursor
1.649	**1.6**	0.935	IPI00769165	Tax_Id=10116 similar to histidine-rich glycoprotein
1.565	**1.516**	0.934	IPI00191789	Tax_Id=10116 Histidine-rich glycoprotein
0.988	0.955	**0.932**	IPI00209744	Tax_Id=10116 Angiotensinogen precursor
1.845	1.778	0.929	IPI00190701	Tax_Id=10116 Apolipoprotein E precursor
2.304	**2.206**	0.923	IPI00199695	Tax_Id=10116 Serine (Or cysteine) peptidase inhibitor, clade F, member 2
1.439	**1.375**	0.921	IPI00201347	Tax_Id=10116 Histidine-rich glycoprotein 1
4.4	**4.187**	0.917	IPI00191715	Tax_Id=10116 Alpha-1-acid glycoprotein precursor
3.305	3.088	0.901	IPI00205036	Tax_Id=10116 Alpha-2-globin chain
0.987	**0.941**	0.883	IPI00195148	Tax_Id=10116 Liver carboxylesterase 1 precursor
1.657	1.449	**0.868**	IPI00230897	Tax_Id=10116 Hemoglobin subunit beta-1
0.996	**0.859**	0.841	IPI00194583	Tax_Id=10116 similar to Apolipoprotein C-II precursor
0.579	**0.405**	**0.831**	IPI00555299	Tax_Id=10116 paraoxonase 1
0.71	0.774	0.794	IPI00363974	Tax_Id=10116 Gelsolin
1.538	**1.266**	0.793	IPI00231423	Tax_Id=10116 C9 protein

3. Validation of Haptoglobin as a Biomarker in Virally Inducible Models of T1D

Validation of haptoglobin as a predictive biomarker of T1D requires analyzing its serum trends to correlate them with disease outcome in the pIC + KRV model. To this end, we performed two validation experiments in which we quantified the changes in serum haptoglobin levels over time by ELISA and western blot. In the first validation study, we wanted to confirm that serum haptoglobin levels were predictive of diabetes outcome with pIC + KRV and not merely with virus infection. BBDR rats were randomized to one of four treatment groups: PBS alone, pIC alone, pIC + KRV or pIC + H-1. KRV and H-1, both parvoviruses, share >80% sequence homology within their structural proteins, and 100% homology within their nonstructural proteins. H-1, however, when injected alone or with pIC, does not cause diabetes in BBDR rats [52]. Therefore, if haptoglobin levels truly correlated with diabetes, its serum profile in the pIC + KRV model would be different from its profile in the pIC + H-1 model. In this experiment rats were tail bled on indicated days, and sera stored for further experimentation.

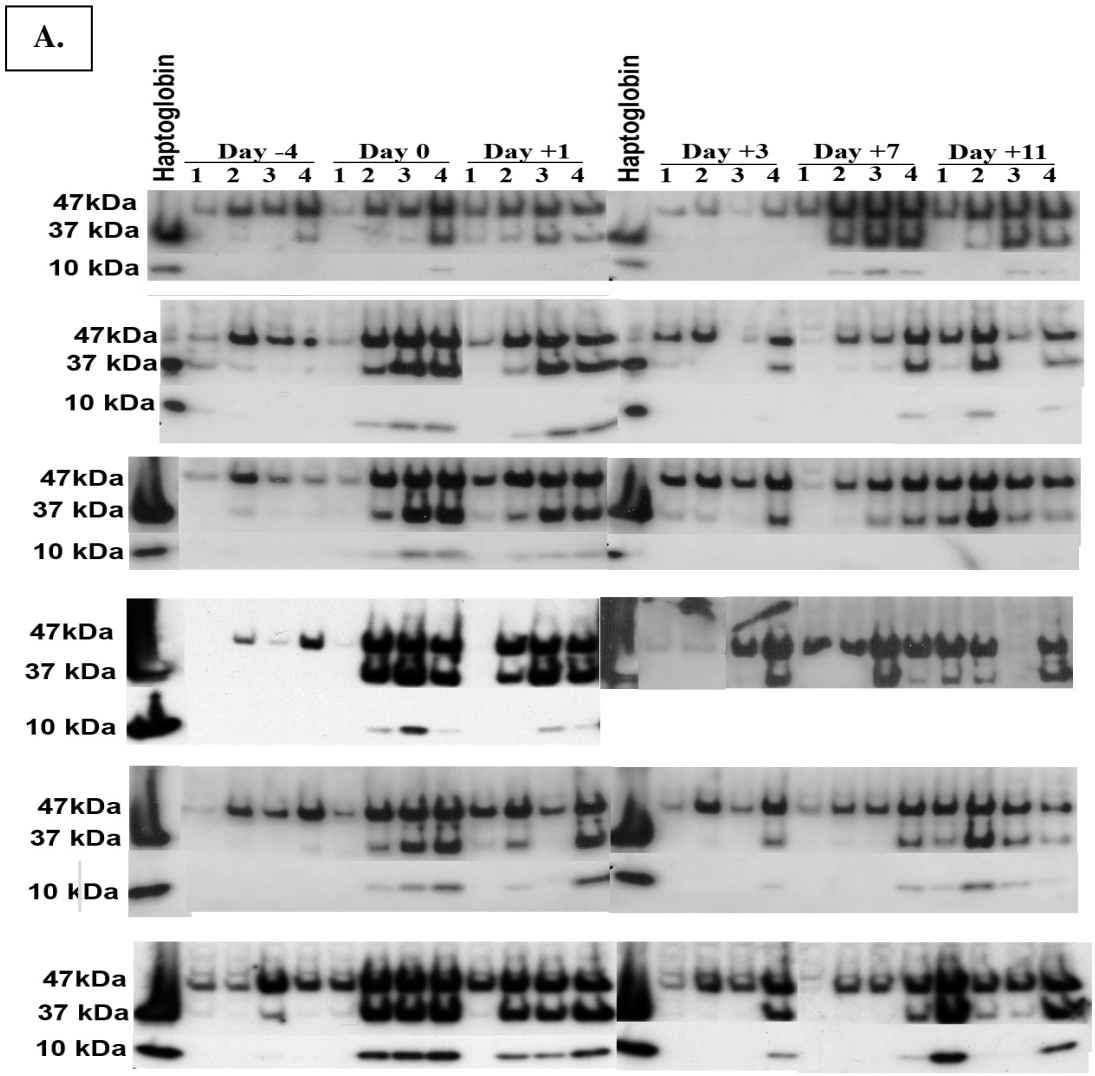
In our second validation experiment, we wanted to confirm that type of bleed did not contribute to the differential selection of haptoglobin as a biomarker. BBDR rats were randomized to the same four groups, PBS alone, pIC alone, pIC + KRV or pIC + H-1, but were terminally cardiac bled on indicated days. Because many of the differentially expressed proteins identified by 2D gel and iTRAQ analyses included proteins involved in hemolysis and heme scavenging pathways, we were concerned about the extent to which the method of bleeding rats might have promoted hemolysis in our samples, thereby contributing to the selective identification of heme associated proteins. Cardiac puncture, in our experience, is

the method least likely to rupture red blood cells and promote hemolysis in samples. Therefore, the first two validation studies employed different bleeding techniques to ensure that the differential expression of haptoglobin in the disease model was not due to an artifact of sample collection.

4. Western Blot Analysis of Serum Haptoglobin Expression

Western blot analysis of undepleted serum samples was performed to validate the results obtained in the 2D gel and iTRAQ experiments. **Figure 2** depicts representative western blot data from the pIC + KRV tail bleed (**Figure 2a**) and cardiac puncture (**Figure 2b**) validation studies. All blots identified three bands in each sample: (1) prohaptoglobin (47kDa), the uncleaved protein that normally circulates in the body even in the absence of infection, and (2) the beta chain (37kDa) and (3) alpha chain (10kDa) of the cleaved, activated haptoglobin molecule. On day 0 and day +1, in both the tail bleed and cardiac puncture experiments, western blot analysis demonstrated an increase in all three bands in the pIC alone and pIC + KRV treated rats compared with rats treated with PBS alone. Serum collected on day 0 corresponds to rats subjected to three consecutive days of pIC treatment (prior to virus infection), while serum from day +1 corresponds to one day following the virus treatment. The strength of all three bands waned in the days subsequent to day +1, although levels remained highest in the pIC + KRV group. This decrease in prohaptoglobin and activated haptoglobin in the serum appears to correspond with the serum clearance of pIC, which can be eliminated from the body 24-48 hours following i.p. injection (Dr. Danny Zipris, personal communication).

Figure 2. Western Blot Analysis of Serum Haptoglobin Levels



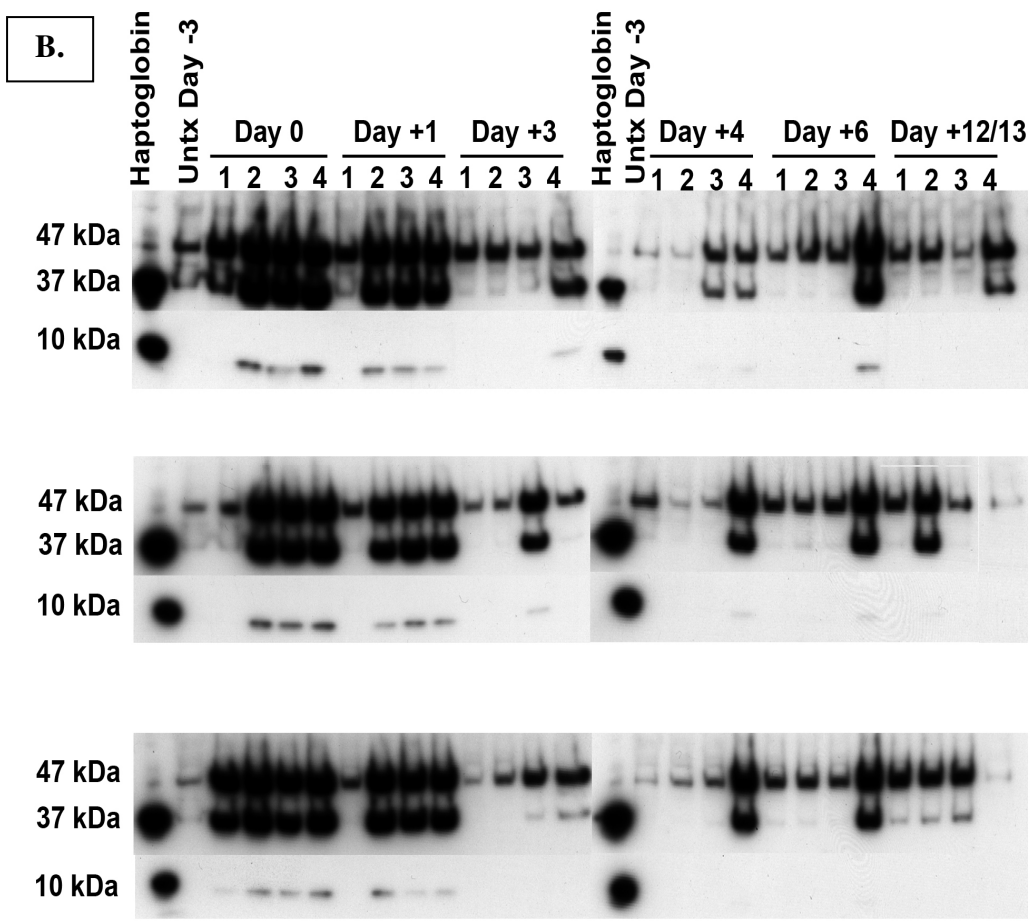


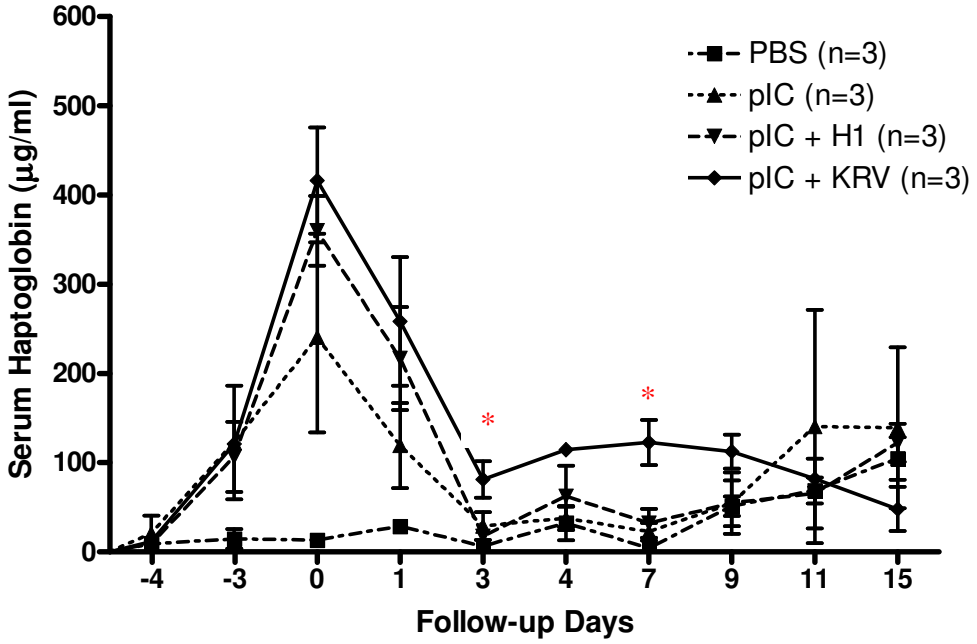
Figure 2. Western blot analysis of undepleted sera from rats treated with PBS alone (1), pIC alone (2), pIC + H-1 (3) or pIC + KRV (4). Protein concentration was assessed by the Bradford assay and equal protein was loaded in each well. Rows represent different rats in each treatment group. Rats were tail bled (A) or cardiac bled (B) at indicated time points. Purified rat haptoglobin is used as a positive control.

5. ELISA Quantitation of Serum Haptoglobin Levels

Serum haptoglobin levels were next quantified by ELISA for each of the two validation studies. In sera from tail bleeds or cardiac punctures of rats treated with PBS alone, pIC alone, pIC + KRV or pIC + H-1, we observed similar trends in serum haptoglobin levels (**Figure 3**). Although the total amount of haptoglobin was lower in the sera harvested from tail blood than cardiac blood, perhaps due to increased clotting in tail bled rats lowering their free serum haptoglobin levels, the overall changes in the haptoglobin levels over time in each of the treatment groups appeared remarkably similar. In fact, in both studies, three consecutive days of pIC treatment induced a surge in serum haptoglobin levels in the pIC alone, pIC + KRV and pIC + H-1 groups (serum haptoglobin levels depicted on day 0 for the virus treated groups are indicative of the effects of pIC alone at that point, as KRV or H-1 viruses were injected following the bleed).

Figure 3. Serum Haptoglobin Levels from Rats Undergoing Diabetes Induction

A.



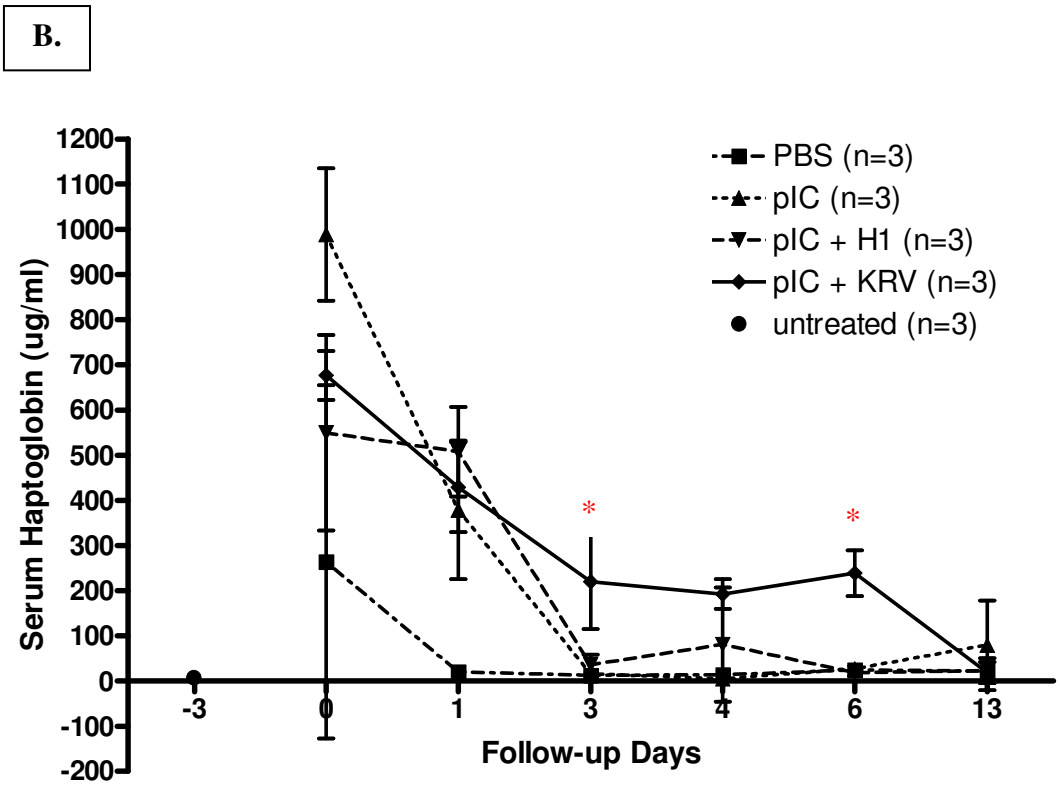


Figure 3. Serum haptoglobin levels determined by ELISA in BBDR rats treated with PBS alone, pIC alone, pIC + H-1 and pIC + KRV. Serum samples were obtained from rats that were (A) tail bled or (B) cardiac bled at indicated time points, and were run in duplicate against a standard curve of purified rat haptoglobin. ANOVA followed by Bonferroni comparison of groups was used to determine differences in serum haptoglobin levels between groups for any given time point. (*$p < 0.05$ between pIC + KRV and pIC + H-1 groups).

In rats that were tail bled, haptoglobin levels spiked on days 0 and +1 in all groups that received pIC relative to rats treated with PBS alone, and declined thereafter, perhaps as a consequence of clearance of pIC from the blood stream. This surge and decline in total protein levels was also observed in the western blot analysis. In contrast, serum haptoglobin levels remained significantly elevated only on days +3 and day +7 in the pIC + KRV treated rats when compared with the pIC + H-1 treated rats (**Figure 3a, $p<0.05$**). Serum levels on day +4 were also higher in the pIC + KRV than the pIC + H-1 treated rats, but this elevation did not reach statistical significance at the 95% confidence level.

In rats that were cardiac bled, haptoglobin levels, again, spiked on day 0 relative to pretreatment, and declined subsequently. However, serum levels remained significantly elevated on day +3 and day +6 in the pIC + KRV treated rats when compared with the pIC + H-1 treated rats (**Figure 3b, $p<0.05$).** As with tail bled rats, cardiac bled rats had higher haptoglobin levels that were only borderline significant on day +4 in the pIC + KRV group compared with pIC + H-1 group.

6. *Native Gel Analysis of Sera Identifies Hemoglobin:Haptoglobin Complexes in Response to pIC Treatment.*

2D analysis followed by MALDI-TOF-MS/MS had identified a significant increase in both haptoglobin and hemoglobin alpha and beta chains in pIC alone and pIC + KRV treated rats. Since haptoglobin is known to complex with hemoglobin, we asked whether these complexes could be visualized on a native gel. We subjected sera from day +1 of rats treated with PBS alone, pIC alone or pIC + KRV, to native gel analysis followed by excision of all

bands that appeared to be different by gross visualization between any treatment groups. Excised bands were tryptically cleaved and peptides identified by MALDI-TOF MS/MS.

Interestingly, the most significant band that appeared in the native gel of serum samples from day +1 of pIC alone and pIC + KRV treated rats was identified as a complex of hemoglobin alpha chain and haptoglobin (**Figure 4**). This band disappeared in the sera from rats treated with pIC alone on days +3 and +6, but persisted in the sera from rats treated with pIC + KRV on those same days. These results are in concordance with the ELISA results from the first and second validation studies where we observed a persistent rise in the serum haptoglobin levels on days +3 and +6/7 of pIC + KRV treated rats, regardless of method of bleed (tail versus cardiac).

Figure 4. Native Gel Analysis Identified Hemoglobin:Haptoglobin Complexes in the Sera of pIC + KRV Treated Rats

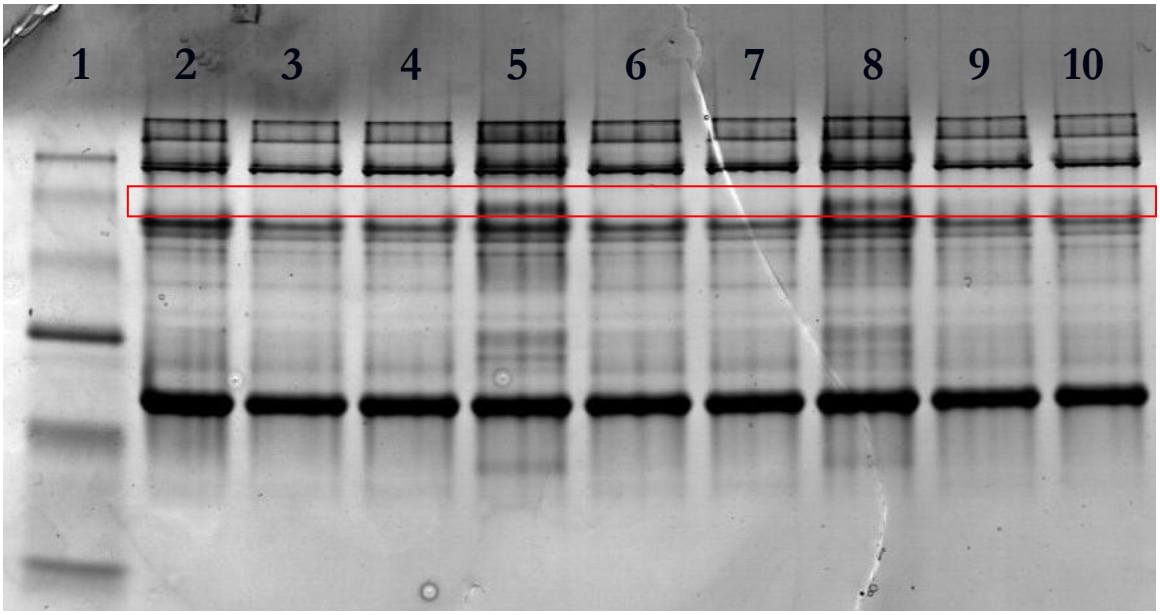


Figure 4. Native gel of sera from BBDR rats treated with PBS alone, pIC alone or pIC+KRV on indicated days. Protein concentration was quantified using the Bradford assay and equivalent concentrations of proteins were loaded into each well. Protein bands (indicated by red box) that differed between groups were excised using a spot cutter from individual lanes. Gel plugs from each lane was tryptically digested and peptides identified by MALDI/MS/MS. Marker (lane 1), PBS only days 0,+3,+6 (lanes 2,3,4), pIC only days 0,+3,+6 (lanes 5,6,7), pIC+KRV days 0,+3,+6 (lanes 8,9,10).

As mentioned previously, activated haptoglobin is a scavenger of free serum hemoglobin that is released as a product of RBC lysis. A variety of infections, including parvoviral infections, can induce RBC hemolysis [95], releasing free hemoglobin into the serum. Because the heme moiety in hemoglobin can be toxic to many cells, haptoglobin is activated in the serum from its pro form and sequesters free hemoglobin molecules, forming hemoglobin:haptoglobin complexes. This complex is taken up by its receptor, CD163, on liver hepatocytes and is subsequently cleared from the circulation [96]. Our native gel data shows that the elevation in serum haptoglobin in response to pIC treatment, as observed on day 0, corresponds with an elevation in the hemoglobin:haptoglobin complex formation in the serum, with a subsequent disappearance of this complex corresponding with the clearance of pIC from the serum. However, in pIC + KRV treatment, the elevation in serum hemoglobin:haptoglobin complexes persisted at least until day +6, the time point when it was last tested (**Figure 4**). These results imply the observed increase in the serum haptoglobin levels, as quantified by ELISA, has a functional consequence of sequestering free hemoglobin in the serum.

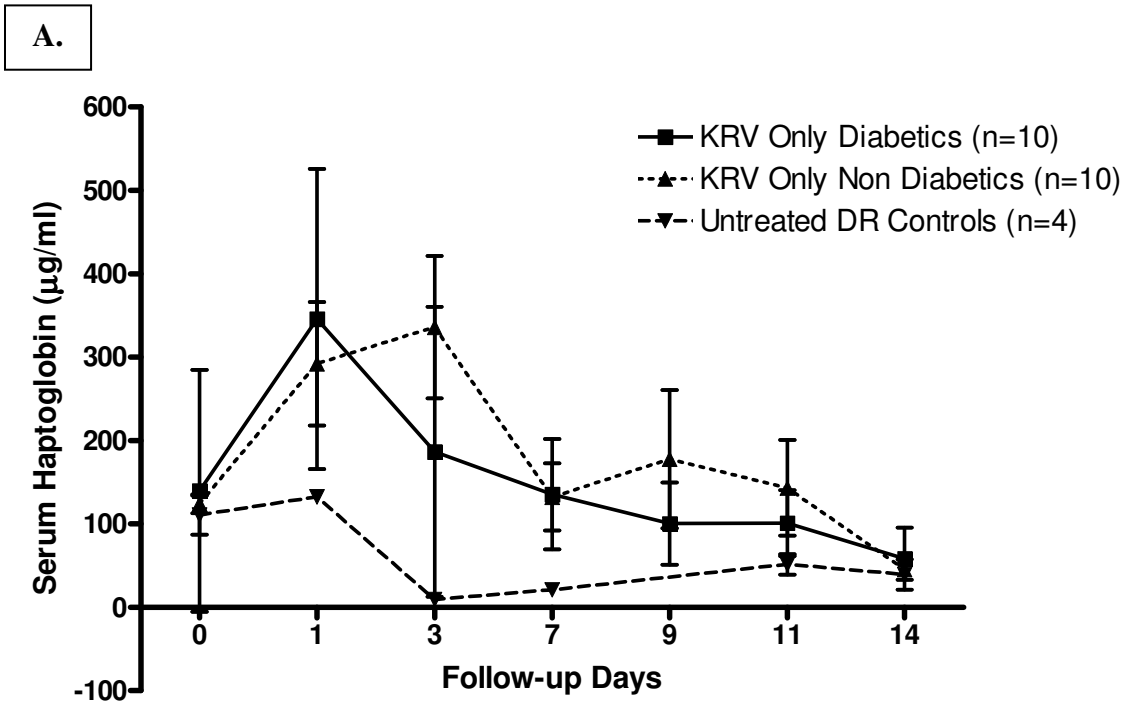
7. Validation of Serum Haptoglobin as a Biomarker Predictive of Diabetes in Multiple Virus Induction Models of the Disease.

Validation of haptoglobin as a predictive biomarker of T1D requires testing the predictive value of the marker in a variety of experimental settings where diabetes penetrance is not complete. For this study, BBDR rats were either untreated or treated with KRV alone (no pIC), and tail bled on indicated days. BBDR rats treated with KRV alone develop T1D with a cumulative frequency of roughly 30% within 21 days [50]. We hypothesized that if

haptoglobin was a sensitive marker for virally induced diabetes, its levels would differ between KRV treated rats that developed diabetes and those that did not. In a similar study, LEW.1WR1 rats were either untreated or treated with RCMV. LEW.1WR1 rats, like BBDR rats, have the susceptible RTI^u MHC haplotype, and develop autoimmune diabetes spontaneously and in response to immune perturbants such as pIC, bacterial lipopolysaccharide, and regulatory T cell depletion [97]. In addition, LEW.1WR1 rats develop T1D in response to RCMV infection, with the same frequency and predictable time course as do BBDR rats in response to KRV (personal communication with J.P. Mordes). In addition, this rat model utilized both a different virus and rat strain to test the sensitivity of our putative serum biomarker, haptoglobin, in predicting T1D. Because haptoglobin levels by Western blot and ELISA analyses gave concordant results, these subsequent studies utilized ELISA alone.

In BBDR rats treated with a single i.p. dose of KRV alone, 42% (10 of 24) of the rats became diabetic within 17 days. Serum haptoglobin levels, as determined by ELISA, increased earlier in the animals that became diabetic than those that did not, however, there was no statistical difference in the haptoglobin levels between rats that became diabetic compared with those that did not at any time point (**Figure 5a**). Haptoglobin levels of KRV treated rats, regardless of diabetes status, were always significantly higher than untreated controls for all time points following infection, indicating that KRV does promote an increase in serum haptoglobin levels.

Figure 5. Serum Haptoglobin Levels in the Virus Only Models of Diabetes Induction



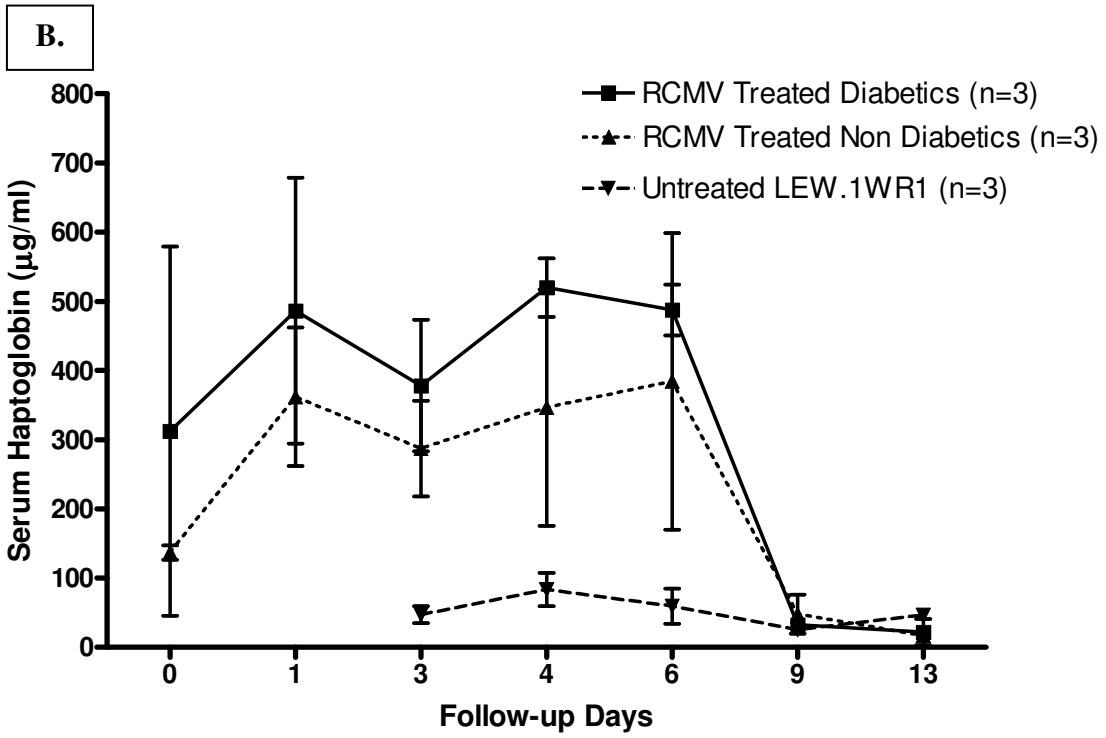


Figure 5. Serum haptoglobin levels determined by ELISA in BBDR rats treated with (A) KRV alone or (B) LEW1.WR1 rats treated with RCMV alone and tail bled on indicated days. ANOVA followed by Bonferroni comparison of groups was used to determine differences in serum haptoglobin levels between groups for any given time point. There were no significant differences in serum haptoglobin levels between diabetic and non-diabetic KRV or RCMV treated rats on any given day.

In LEW1.WR1 rats treated with a single dose of RCMV, 22% (3 of 11) became diabetic within 16 days. Although there was no significant difference in haptoglobin levels between RCMV treated rats that became diabetic compared with those that did not, RCMV treated rats that went on to become diabetic did have higher haptoglobin levels at time points following treatment with the virus when compared with untreated controls (**Figure 5b**). We surmise that the lack of a statistically significant difference in serum haptoglobin levels in diabetic versus non-diabetic rats in the KRV only and RCMV only protocols may be due to the lack of the strong, synergistic effect on liver acute phase protein upregulation induced by the addition of a TLR ligand in these models, as is observed when pIC is administered with KRV.

Summary

We utilized 2D gel electrophoresis followed by MALDI-TOF MS/MS and iTRAQ proteomic profiling techniques to identify early serum biomarkers that could predict the onset of T1D in a virally inducible model of the disease. Both of these global proteomic techniques identified haptoglobin as a potential biomarker which deserved further evaluation for its predictive value. We utilized four independent virus induction models with variable expression of T1D to assess the predictive value of haptoglobin. We found that haptoglobin levels were significantly higher on days +3 and +6/+7 in rats that became diabetic when treated with pIC + KRV, when compared with rats treated with pIC + H-1. Interestingly, the functional role of increased serum haptoglobin levels was confirmed by the native gel analysis which revealed an increase in hemoglobin:haptoglobin complexes in the sera of pIC + KRV treated rats on days +3 and +6, when compared with rats treated with PBS or pIC

alone. We did not observe a difference in haptoglobin levels between diabetics and non-diabetics treated with KRV alone or RCMV alone. The lack of a differential expression of haptoglobin between diseased and non-diseased groups in these virus only models appears to lower the sensitivity of haptoglobin as a biomarker in cases where the pentrance of T1D induced by viruses is lower than in the pIC + KRV model.

CHAPTER IV: LEPTIN MEDIATED PROTECTION FROM VIRALLY INDUCIBLE TYPE 1 DIABETES

Introduction

The adipocyte derived hormone, leptin, has been shown to have pleotropic effects on a variety of tissues. Its greatest relevance to autoimmune diabetes is with regards to its effects on the beta cell and the immune system. In both *in vitro* and *in vivo* models of type 2 diabetes, investigators have demonstrated that hyperleptinemia can inhibit glucose stimulated insulin secretion from beta cells, decrease beta cell TG content and decrease beta cell apoptosis in type 2 diabetes prone rodents [70, 73, 98, 99]. In leptin deficiency states of both mice and humans, the development of the immune system is negatively affected, with humans and mice becoming predisposed to a variety of infections [76]. In contrast, direct infusion of leptin intrathecally can have a suppressive effect on the immune system [100]. We hypothesized that serum hyperleptinemia could play a dual role in both cytoprotecting beta cells and skewing the immune response away from an autoimmune attack on the beta cell in the pIC + KRV model. In the following studies we analyzed the role of hyperleptinemia in affecting diabetes outcome in the pIC + KRV model.

Results

1. *AdLeptin Protects BBDR Rats from Virally Induced T1D.*

To determine whether hyperleptinemia could protect weanling BBDR rats (21-25 days old) from virally induced autoimmune diabetes, we pretreated male and female rats with a single i.v. dose of $1x10^{10}$ infectious units per 0.5ml PBS per 100 gram body weight of either

AdLeptin, or AdBetagal, or the volume equivalent of PBS, followed by the pIC + KRV induction protocol (**Figure 6**). Additional control groups in this study included rats treated with AdLeptin, AdBetagal or PBS alone without the pIC + KRV induction. All rats were followed for up to 45 days post KRV treatment and monitored for the incidence of T1D. Rats that became diabetic were treated with a subcutaneous insulin pellet on the second day of high blood glucose and remained on the pellet until they were sacrificed at end of the study period.

At 45 days post KRV, the cumulative incidence of T1D in the PBS + pIC + KRV group was 90% (10% diabetes free survival, n=10), in line with incidence estimates previously observed in our lab. We observed a significantly greater diabetes free survival in the AdLeptin pretreated rats (>90%, n=22), compared with AdBetagal (50%, n=16), or PBS pretreated rats (10%, n=10) (**Figure 7**). None of the rats in the AdLeptin, AdBetagal or PBS alone treatment groups developed diabetes during the study period (data not shown).

We determined that the average time to diabetes onset in the PBS + pIC + KRV group was 14.3 days, while in the AdBetagal + pIC + KRV it was 15.6 days, which was not statistically different between these groups. This finding is interesting because although only 50% of AdBetagal pretreated rats developed diabetes following virus induction, pretreatment with AdBetagal did not appear to alter the average time to latency in those rats that did become diabetic following pIC + KRV. More importantly, of the two rats that became diabetic with AdLeptin pretreatment, latency to onset was delayed to 24.5 days which was significantly different from both the PBS and AdBetagal pretreated groups ($p<0.001$ and $p<0.01$, respectively).

Figure 6. Schematic of the Type 1 Diabetes Protection Protocol

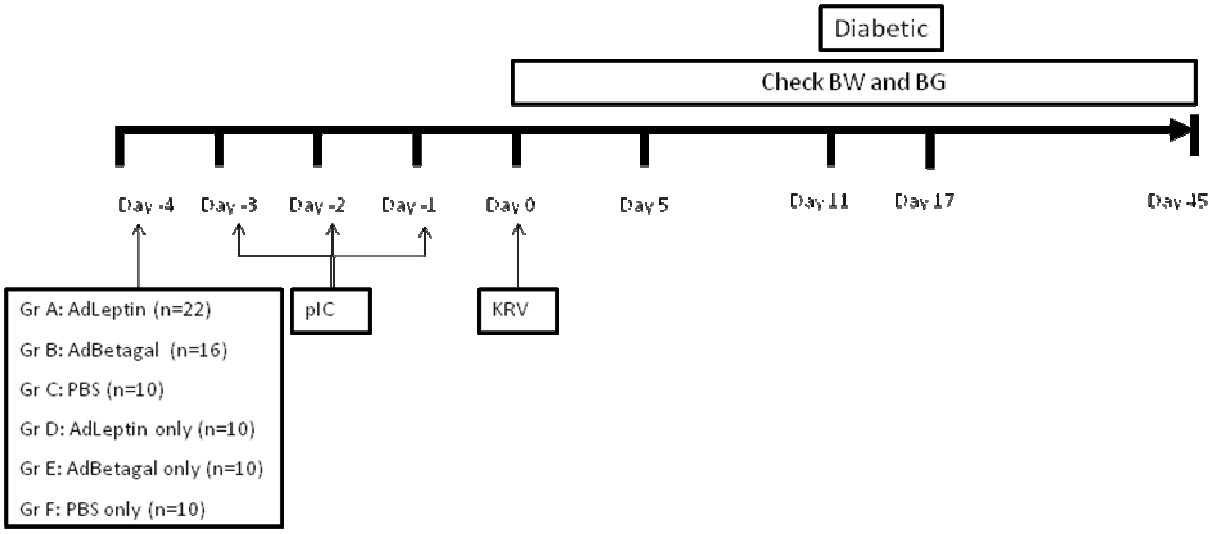


Figure 6. Male and female BBDR rats aged 21-25 days old were treated on day -4 with $1x10^{10}$ infectious units/0.5 ml/100 g body weight i.v. of AdLeptin, AdBetagal or an equal volume of PBS. One subset of rats in each group was further treated with 1µg/g body weight of pIC i.p. for 3 consecutive days (days -3, -2, and -1) followed by $1x10^{7}$pfu of KRV i.p. on the fourth day (day 0). Rats were followed for up to 45 days for the development of diabetes. Body weights and blood glucoses were checked at least three times per week, and any rats exhibiting glycosuria were tested for blood glucose levels on two consecutive days. Any rats that became diabetic were treated with an insulin pellet subcutaneously and followed until the end of the study period.

Figure 7. AdLeptin Protects BBDR Rats from pIC + KRV Induced Type 1 Diabetes

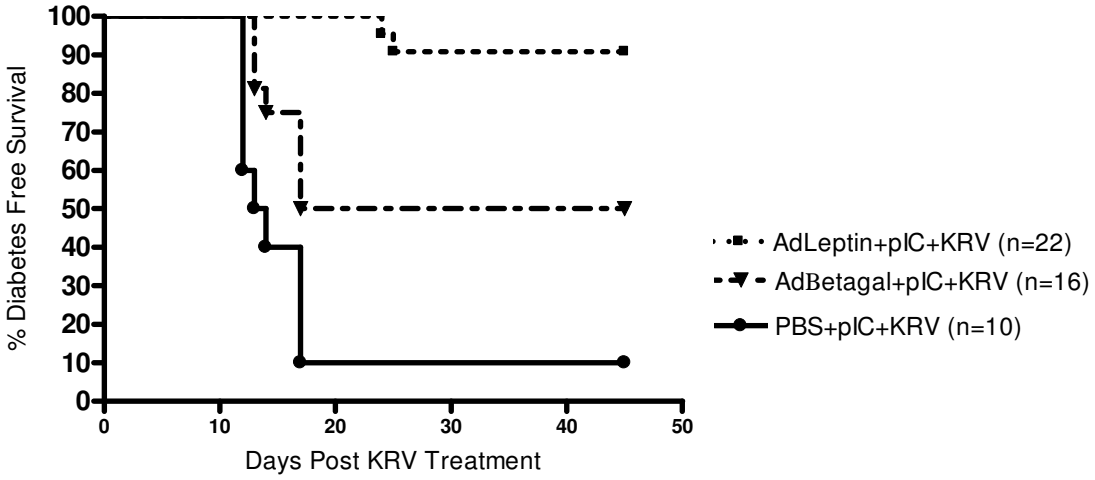


Figure 7. A Kaplan Meier survival curve depicts the differences in the diabetes free survival time between treatment groups. Diabetes free survival is defined as the time until the first of two high blood glucose levels > 250 mg/dl on two consecutive days. Data are pooled from two independent experiments. Statistical analysis of survival differences between treatment groups was based on the Log Rank test: AdLeptin vs AdBetagal ($p=0.0026$); AdLeptin vs. PBS ($p<0.005$); AdBetagal vs. PBS ($p=0.01$).

Both male and female rats pretreated with AdLeptin exhibited significantly lower body weights relative to other treatment groups, but still experienced linear growth during the study period (**Figures 8 and 9**). Body weights of AdLeptin pretreated male and female rats were as low as 65% of the weight of AdBetagal or PBS pretreated rats within 14 days following KRV, and remained at 85% of the body weight of the other groups by 45 days. Body weights among diabetics did not appear significantly different from non-diabetics in any group since rats were administered insulin pellets following their second high blood glucose reading and thereby did not experience further weight loss. Although we did not measure the dietary intake of AdLeptin pretreated rats, the lack of weight gain exhibited by these rats may be attributable to the reported anorexigenic effects of leptin.

Retrospective analysis of the mean non-fasting blood glucose levels among non-diabetic AdLeptin treated rats revealed that non-fasting blood glucose levels in leptinized rats were lower during the first 14 days following KRV treatment when compared with non-diabetics in all other treatment groups (**Figure 10**). These results reached statistical significance at 95% confidence level on days 3, 7, 10 and 14 of follow-up. The drop in blood glucose among AdLeptin treated rats corresponded with their lack of weight gain during this time period and may also be attributable to the anorexigenic effects of leptin. We did not notice any observable differences in the appearance or activity of AdLeptin treated rats compared with other groups at any time during the study. There was no significant difference in the non-fasting blood glucose levels among non-diabetics in any of the other treatment groups. No additional gender based differences in non-diabetic blood glucose levels were observed between any of the treatment groups.

Figure 8. AdLeptin+pIC+KRV Treated Male BBDR Rats Fail to Gain Weight

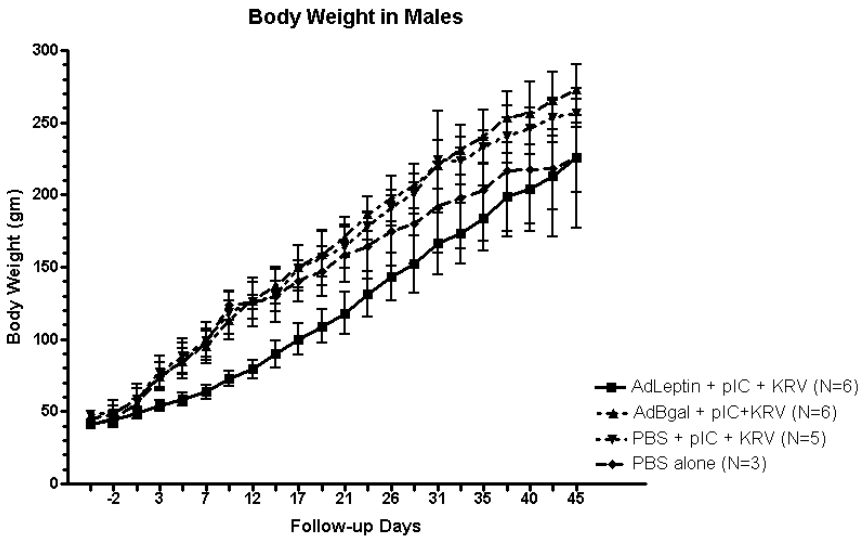


Figure 8. Body weights in grams of a subset of male BBDR rats pretreated with AdLeptin, AdBetagal or PBS prior to pIC + KRV, or treated with PBS alone. Mean weight and SD are represented for each group. Body weights include those of diabetic and non-diabetic rats in the first three groups.

Figure 9. AdLeptin+pIC+KRV Treated Female BBDR Rats Fail to Gain Weight

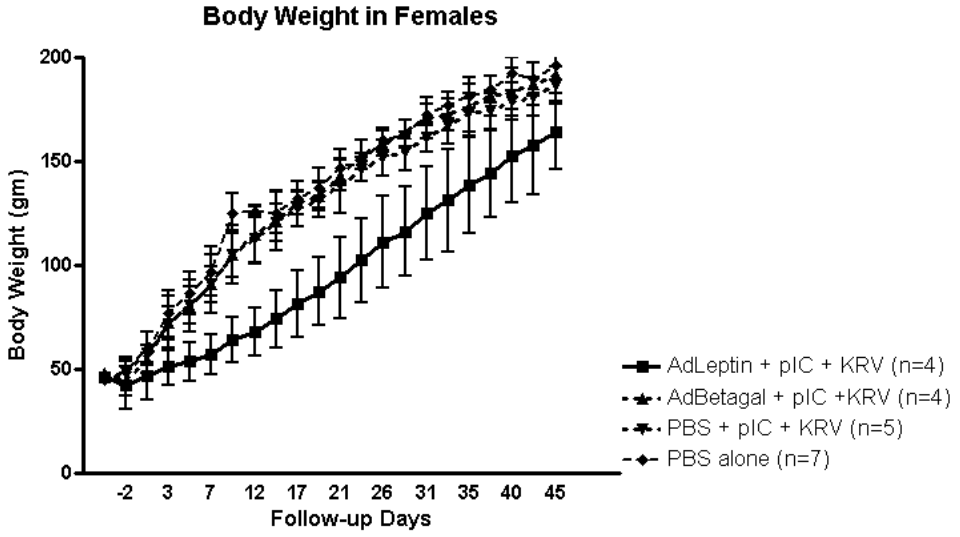


Figure 9. Body weights in grams of a subset of female BBDR rats pretreated with AdLeptin, AdBetagal or PBS prior to pIC + KRV, or treated with PBS alone. Mean weight and SD are represented for each group. Body weights include those of diabetic and non-diabetic rats in the first three groups.

Figure 10. Mean Blood Glucose Levels in AdLeptin Pretreated Rats Are Lower During the First Two Weeks of Follow-up

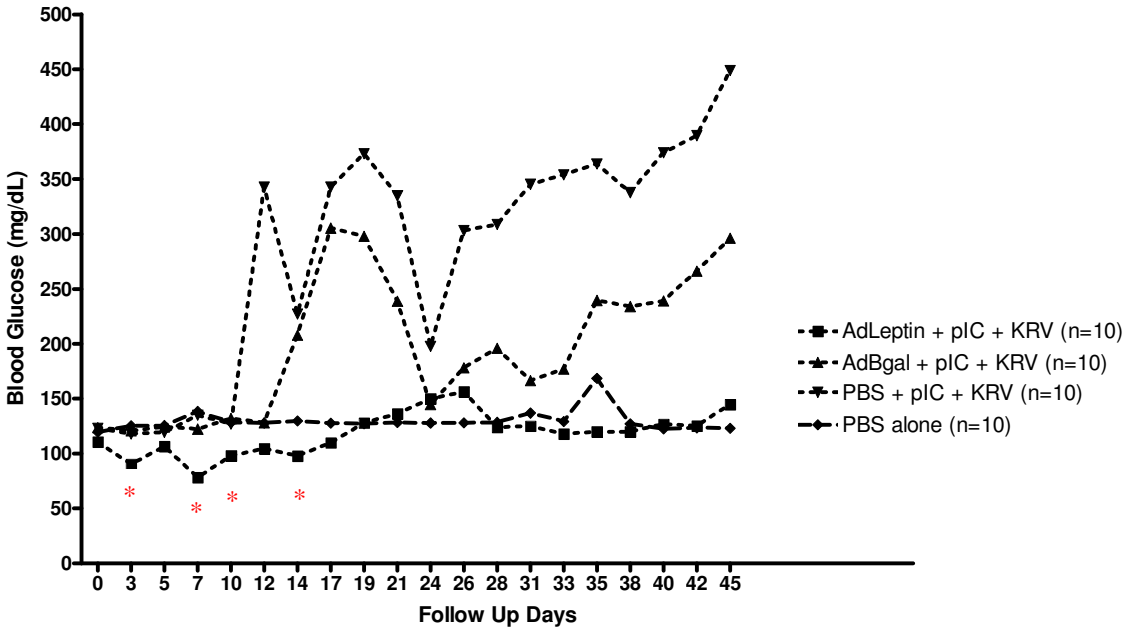


Figure 10. Mean blood glucose levels were recorded 3 – 4 times a week for a subset of rats in the protocol. Mean blood glucose values in each group include diabetics and nondiabetics in the group. Due to high variances in the blood glucose levels within a group (due to inclusion of diabetic and non-diabetic rats), we did not plot the SD for these blood glucose values. ANOVA analysis followed by Bonferroni comparison of blood glucose levels between groups on days 3, 7, 10 and 14 demonstrated statistically significant decreases (*$p < 0.05$) in blood glucose levels between the AdLeptin + pIC + KRV treated group and all other groups.

We also measured serum leptin levels by RIA in this study on: day 0 (four days after adenovirus or PBS pretreatment and day of KRV - serum harvested prior to KRV infection), day 14, and day 45. The mean serum leptin levels in the AdLeptin pretreated group was 35 ng/ml ± 6.7 on day 0, which declined to 21 ng/ml ± 12 by day 14 and to 4 ng/ml ± 2 by day 45 (**Figure 11**). In contrast, mean serum leptin levels in the AdBetagal and PBS pretreated groups remained at 1.0 – 1.2 ng/ml for each of those time points. Among rats treated with PBS alone (not undergoing KRV induction) mean serum leptin levels were 1.7 ng/ml ± 0.7 and 1.4 ng/ml ± 0.5 on day 0 and day 14, respectively, and increased to 3.0 ng/ml ± 1.8 by day 45. These data demonstrate that AdLeptin pretreatment was able to generate a hyperleptinemic state by raising mean serum leptin levels to 35 times normal at the time of KRV treatment. These serum leptin levels remained as high as six times normal at the end of the study period. Mean serum leptin levels of rats treated with AdLeptin alone were not significantly different from levels in the AdLeptin + pIC + KRV treated group at all time points tested (**Table 8**).

Of the two AdLeptin pretreated rats that became diabetic during the study (both males), one had lower serum leptin levels than average (18.6 ng/ml) on day 0, and subsequently declined to 3.0 ng/ml by day 45. However, the second diabetic male had serum leptin levels in range with the other rats (31 ng/ml on day 0, 10 ng/ml on day 14, and 4 ng/ml on day 45). Therefore, we conclude that although the hyperleptinemic state was unable to prevent diabetes in two AdLeptin pretreated rats, all other hyperleptinemic rats were protected from T1D.

Figure 11. AdLeptin Induces Serum Hyperleptinemia in BBDR Rats

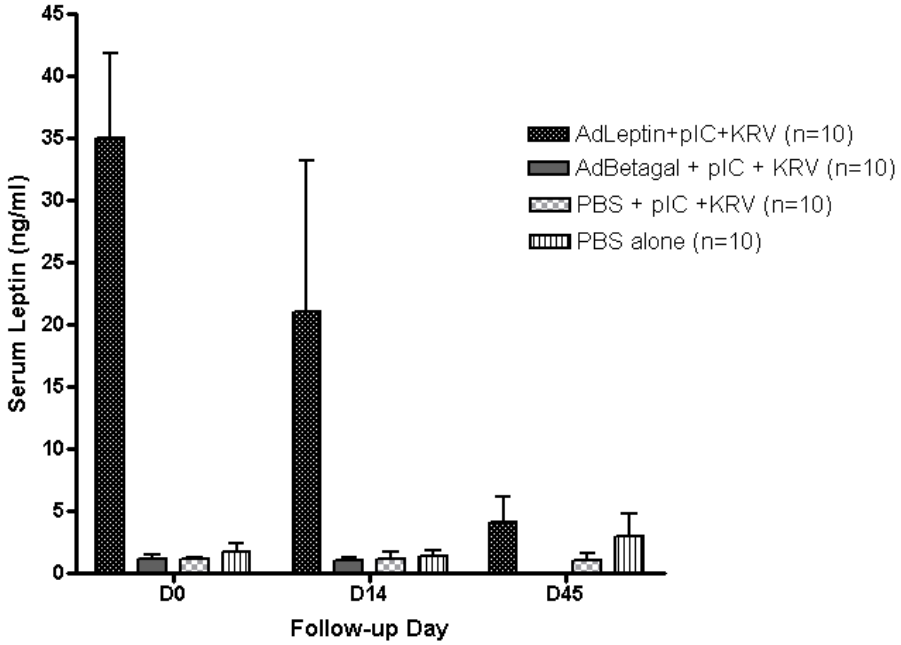


Figure 11. BBDR rats were tail bled on day 0, day 14 and day 45 of follow-up for assessment of serum leptin levels by RIA. All samples were tested in duplicate. The mean and standard deviations for each group are represented. Data includes all diabetics and non-diabetics in each group.

Table 8. Serum Leptin Levels by Gender and Diabetes Status in Treatment Groups

Treatment Group	**Day 0**		**Day 14**		**Day 45**	
AdLeptin + pIC + KRV	Diabetics (n)	Non-diabetics (n)	Diabetics (n)	Non-diabetics (n)	Diabetics (n)	Non-diabetics (n)
Males	24.8 (2)	39.2 (4)	5.9 (2)	29.0 (4)	3.4 (2)	14.9 (4)
Females		36.0 (4)		20.8 (4)		2.7 (4)
AdBetagal + pIC + KRV						
Males	0.7 (2)	1.3 (4)	1.0 (2)	1.2 (4)	NA	NA
Females	1.2 (3)	1.4 (1)	1.0 (3)	0.5 (1)	NA	NA
PBS + pIC + KRV						
Males	1.1 (5)		1.3 (5)		0.7 (5)	
Females	1.1 (4)	1.34 (1)	1.0 (4)	0.99 (1)	1.3 (4)	1.51 (1)
AdLeptin Alone						
Males		23.3 (6)		23.4(4)		12.8 (4)
Females		41.4 (6)		33.3 (4)		7.8 (6)
AdBetagal Alone						
Males		1.9 (3)		1.3 (3)		NA
Females		1.3 (7)		1.03 (7)		NA
PBS alone						
Males		2.3 (3)		1.5 (3)		5.4 (3)
Females		1.6 (7)		1.4 (7)		2.0 (7)

Table 8. Mean serum leptin levels in male and female diabetic and non-diabetics in each treatment group in the study. Serum leptin levels were calculated by RIA, and all samples were tested in duplicate. Data for rats in the AdBetagal + pIC + KRV and AdBetagal alone groups on day + 45 were not available (NA = not available). The sample size used to calculate the mean leptin level in each subgroup is indicated in parentheses.

In a subset analysis we retrospectively compared the serum leptin levels of all diabetics with non-diabetics in either the AdBetagal + pIC + KRV or the PBS + pIC + KRV groups on day 0. We discovered that serum leptin levels in rats that would go on to become diabetic in either group were significantly lower (by 20%) than rats that would remain non-diabetic until the end of the study. This difference was further accentuated when we compared all the diabetics with non-diabetics in the AdBetagal + pIC + KRV, the PBS + pIC + KRV, and PBS alone groups (**Figure 12**). In this comparison, serum leptin levels on day 0 in rats that would go on to become diabetic were significantly lower (by 33%) than rats that would remain non-diabetic until the end of the study ($p=0.004$). There were no significant differences, however, in the serum leptin levels on day 0 of AdBetagal + pIC + KRV treated rats that would go on to become diabetic compared with those that would not.

Sera collected for testing leptin levels were also analyzed for nonfasting serum insulin levels by RIA. There was no difference in nonfasting serum insulin levels between AdLeptin and PBS alone (no pIC + KRV) treated rats on day 0. However in pIC + KRV treated rats, we found that nonfasting serum insulin levels were significantly lower in the AdLeptin pretreated rats on day 0 (day of KRV treatment) when compared with AdBetagal or PBS pretreated rats (**Figure 13**). On day 14 post KRV treatment, among all non-diabetic rats, nonfasting serum insulin levels were also significantly lower in the AdLeptin pretreated group compared with control AdBetagal and PBS pretreated rats, but not significantly different from serum insulin levels of rats treated with PBS alone (no pIC + KRV). These data suggest that the rise in serum insulin levels elicited by pIC + KRV treatment could be suppressed by the hyperleptinemia induced by AdLeptin.

Figure 12. Serum Leptin Levels In Rats That Become Diabetic Are Significantly Lower on Day 0

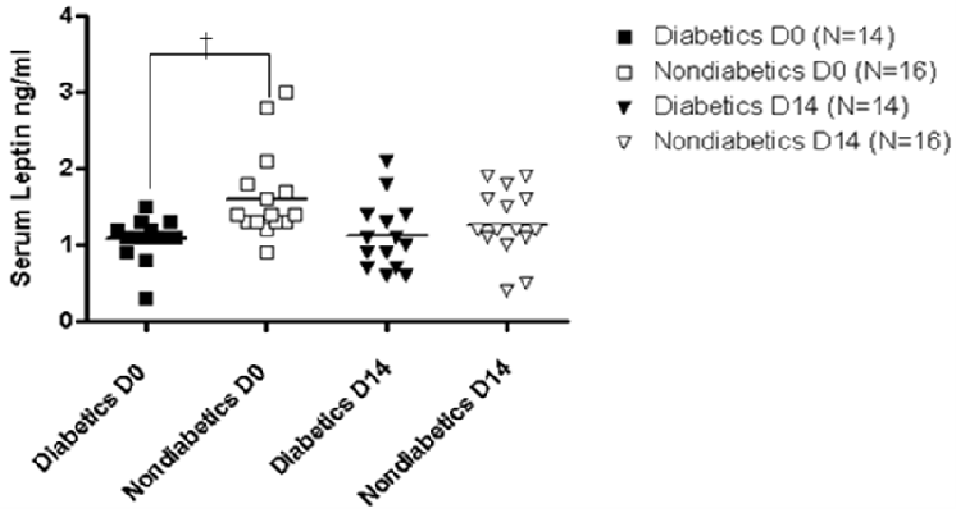


Figure 12. Sera of rats in the AdLeptin + pIC + KRV, Ad Betagal + pIC + KRV or PBS + pIC + KRV groups that would become diabetic or remain non-diabetic were analyzed for leptin levels by RIA on day 0 (day of KRV, rats bled prior to KRV infection), or day 14 following KRV infection. All samples were tested in duplicate, and mean serum values calculated for each sample. Serum leptin levels were significantly lower on day 0 among rats that would become diabetic in any group, than those that would remain non-diabetic during the study period (†p=0.003 based on the Student's T test comparison at the 95% confidence level).

Figure 13. Non-fasting Serum Insulin Levels are Significantly Lower in AdLeptin Treated Rats than in Controls

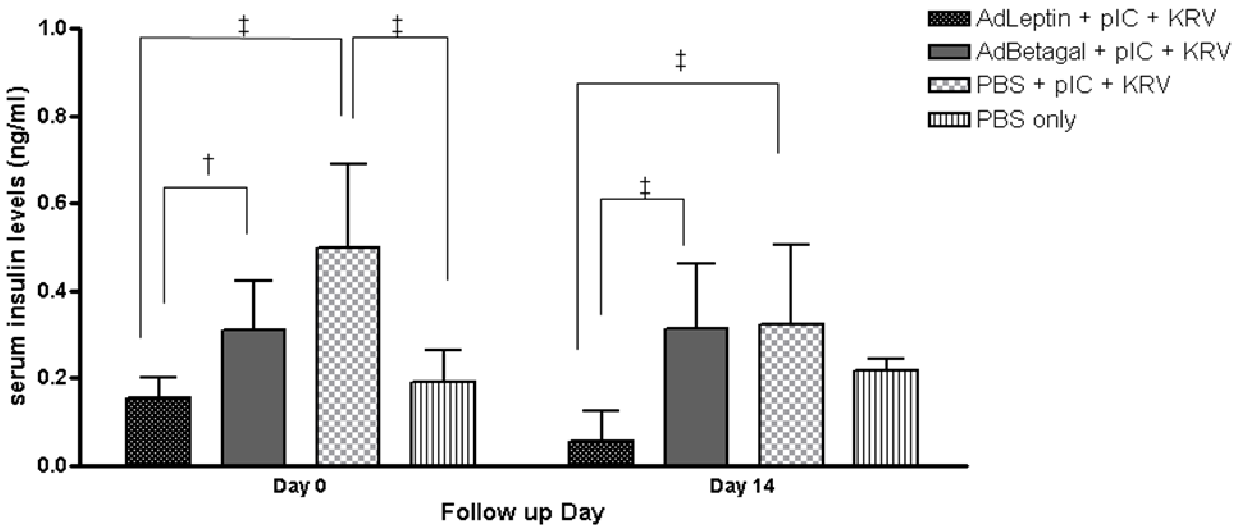


Figure 13. Sera from rats in the AdLeptin + pIC + KRV, Ad Betagal + pIC + KRV, PBS + pIC + KRV or PBS only groups were analyzed for insulin levels by RIA on day 0 (day of KRV, rats bled prior to KRV infection), or day 14 following KRV infection. All samples were tested in duplicate, and mean serum values calculated for each sample. Serum insulin levels were significantly lower in AdLeptin pretreated rats on day 0 and day 14 compared with other groups (†$p<0.05$, ‡ $p < 0.001$ based on an ANOVA followed by Bonferroni comparison of groups). Data from day 14 only represent nondiabetic rats in each group (N=10 for each group on day 0; N=7 for AdBetagal + pIC + KRV on day 14; N=6 for PBS + pIC + KRV on day 14).

To determine if hyperleptinemia altered the infiltration of pancreatic islets by immune cells normally observed with pIC + KRV treatment, we evaluated histologic sections of pancreas harvested from rats at the end of the study. Islets of diabetes-free AdLeptin protected rats were intact with minimal (1+) to no insulitis, and positive insulin and glucagon staining by IHC analysis (**Figure 14**). In contrast, islets from diabetic AdBetagal or PBS pretreated rats appeared as end-stage, involuted islets, with no insulin staining, and central glucagon staining resulting from the absence of beta cells in the islets. There was also no remnant insulitis in the islets of AdBetagal or PBS pretreated rats due to complete beta cell destruction and subsequent departure of beta cell-reactive immune cells from the tissue by 45 days post KRV treatment. Pancreas sections from the two AdLeptin treated rats that became diabetic also revealed predominantly end stage islets, however a few shrunken islets with some remnant insulin staining were also visible (**Figure 15**). No remnant insulitis was present in tissue sections from either of these rats on day 45.

We conclude from this study that hyperleptinemia induced by AdLeptin protects rats from pIC + KRV induced autoimmune diabetes, suppresses the rise in serum insulin levels observed following KRV and prevents the autoimmune infiltration and destruction of beta cells in pancreatic islets. Hyperleptinemia prevented normal body weight gain in both male and female rats pretreated with AdLeptin. However, the rats continued to exhibit linear growth during the entire study period. We were concerned about the effect of the adenovirus vector in conferring some degree of protection from autoimmune diabetes, since 50% of the AdBetagal treated rats did not develop the disease in this study. Although we were surprised to observe a 50% cumulative survival in the AdBetagal group, this survival may be

attributable to the following possibilities: (1) the effect of pretreatment with an adenovirus potentially skewed the adaptive immune response towards the adenovirus and away from a beta cell autoreactive process, (2) the overproduction of beta galatosidase by the adenovirus vector altered the immune milieu in such a way as to prevent beta cell autoreactivity or (3) the infection of hepatocytes by the adenovirus vector altered the innate immune response in a way to prevent beta cell autoreactivity. Additionally, unpublished studies from our lab have shown that order of administration of RCMV and KRV into BBDR rats can alter the incidence and kinetics of T1D in these rats (personal communication, Dr. John Mordes). To address the confounding effects of the adenoviral vector, we next repeated the protection study using alzet pumps to deliver a continuous, high dose of rat recombinant leptin (rrLeptin).

Figure 14. Islets from AdLeptin + pIC + KRV Protected Rats Are Protected from Insulitis and Stain Positively for Insulin

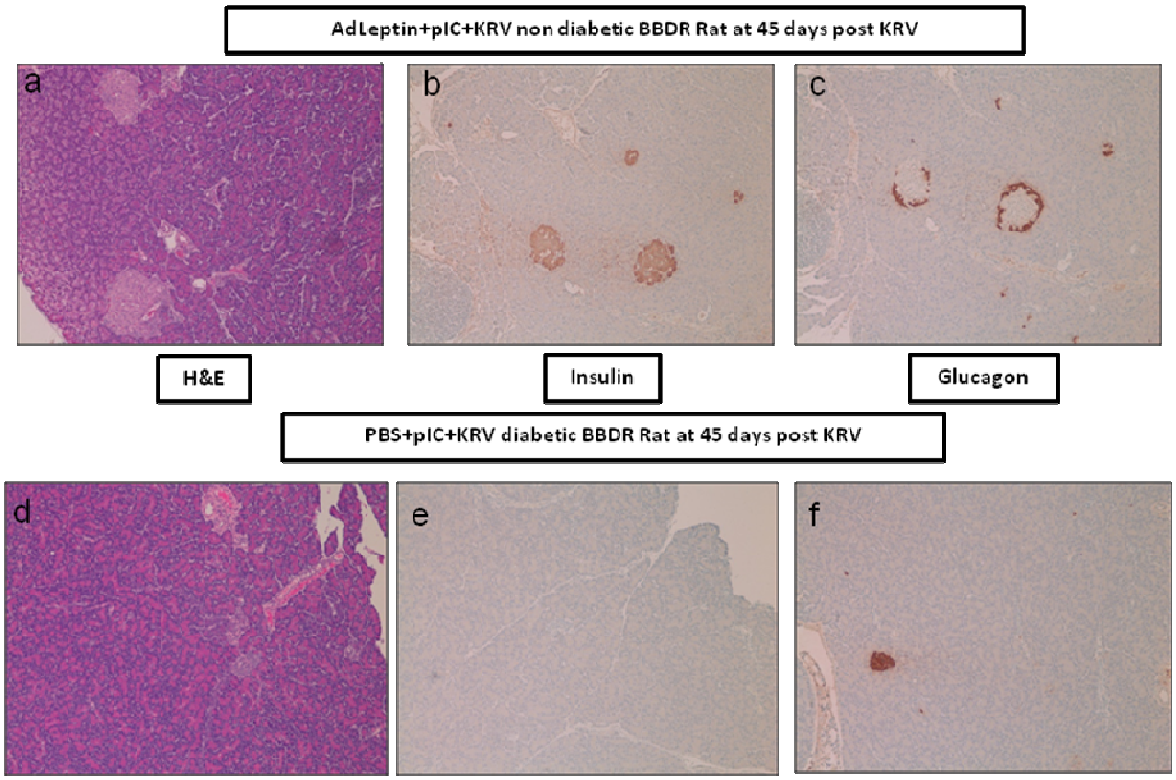


Figure 14. Upper panels: representative pancreas sections from AdLeptin + pIC + KRV treated rat protected from diabetes on day 45 (a) H&E of pancreas demonstrates minimal to no insulitic infiltrate in or around islets (b) insulin staining by IHC demonstrates strong central staining of beta cells producing insulin and (c) glucagon staining by IHC demonstrates strong peripheral staining of alpha cells. Lower panels: representative pancreas sections from day 45 of a diabetic rat treated with PBS + pIC + KRV. (d) H&E demonstrates involuted, shrunken islets and no remnant insulitis (e) no insulin staining by IHC due to complete beta cell destruction and (f) central glucagon staining from alpha cells of involuted islets. All images obtained at 10x magnification.

Figure 15. Islets from Diabetic Rats Treated with AdLeptin + pIC + KRV Demonstrate the Presence of Remnant Insulin Positive Beta Cells

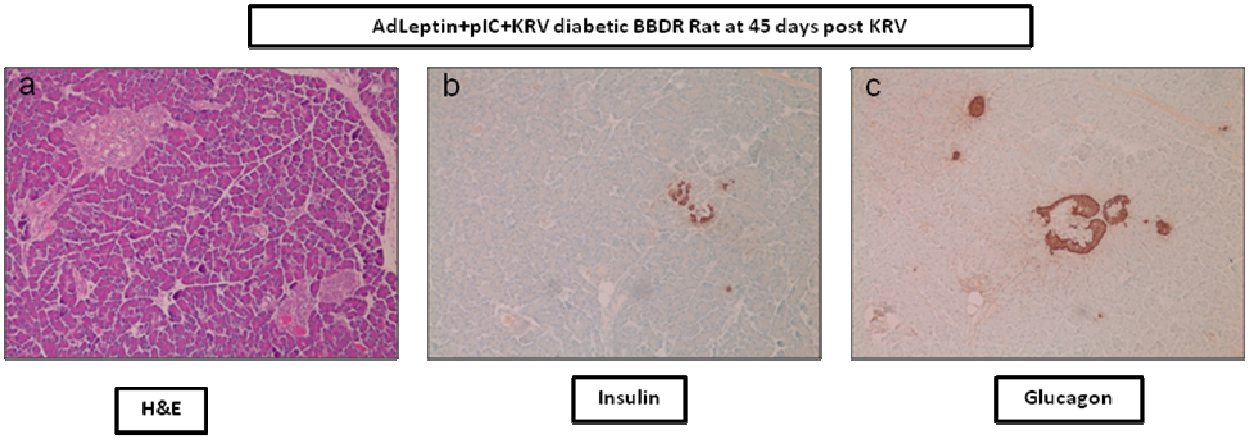


Figure 15. Representative pancreas sections from a diabetic rat on day 45 that was treated with AdLeptin + pIC + KRV. (a) H&E demonstrated predominantly involuted islets, however, a few remnant islets with insulitic infiltrates were still present (b) presence of rare insulin positive beta cells and (c) predominantly central staining of glucagon in involuted islets, but occasional peripheral glucagon staining in islets that continued to stain positive for insulin.

2. Pharmacokinetics of rrLeptin Using Alzet Pumps

We first performed a pharmacokinetic analysis to determine which doses of recombinant leptin (rrLeptin) delivered by alzet pumps would result in similar serum leptin levels achieved using the adenovirus vector. Twenty-one to 25 day old male BBDR rats were treated with either vehicle alone or the following doses of rat recombinant leptin (rrLeptin) delivered using a single alzet pump (model 2001) for 7 days: 0.6 µg/g/day, 1.2 µg/g/day, or 1.5 µg/g/day. Pumps were removed on the seventh day following implantation, and rats were sacrificed on the eighth day of follow-up. Rats were tail bled multiple times for analysis of their serum leptin levels. Body weights and blood glucose readings were also recorded during the eight day follow up period.

Peak serum leptin levels achieved with 1.2 µg/g/day and 1.5 µg/g/day dosages were similar at 27.2 ng/ml and 29.7 ng/ml, respectively, and significantly higher than the peak serum levels achieved with the 0.6 µg/g/day dose (5.5 ng/ml) (**Figure 16**). The average serum leptin level in vehicle control treated rats was 1.0 ng/ml, ranging between 0.9 ng/ml and 1.3 ng/ml during the study period. In rats dosed with rrLeptin at 1.2 µg/g/day and 1.5 µg/g/day, peak levels were observed on day +1 following pump implantation and subsequently declined to 15 ng/ml and 10 ng/ml, respectively. It appeared that the 1.2 µg/g/day dose was as effective or more effective than the 1.5 µg/g/day dose in achieving peak serum leptin levels, preventing weight gain (**Figure 17**) and maintaining low, non-fasting blood glucose levels (**Figure 18**). However, when we compared the serum leptin levels achieved with 1.2 µg/g/day dose per pump on day 4 following pump insertion (15 ng/ml) with the serum leptin levels achieved four days after AdLeptin administration (35

ng/ml) we realized that we would need to install double pumps delivering 1.2 µg/g/day of rrLeptin to achieve similar serum leptin levels as conferred by the adenoviral delivery system. The serum leptin levels achieved using seven day alzet pumps also revealed that the pumps might maintain higher serum levels if they were replaced every five instead of every seven days.

Figure 16. Alzet Pump Delivery of rrLeptin Generates Serum Hyperleptinemia

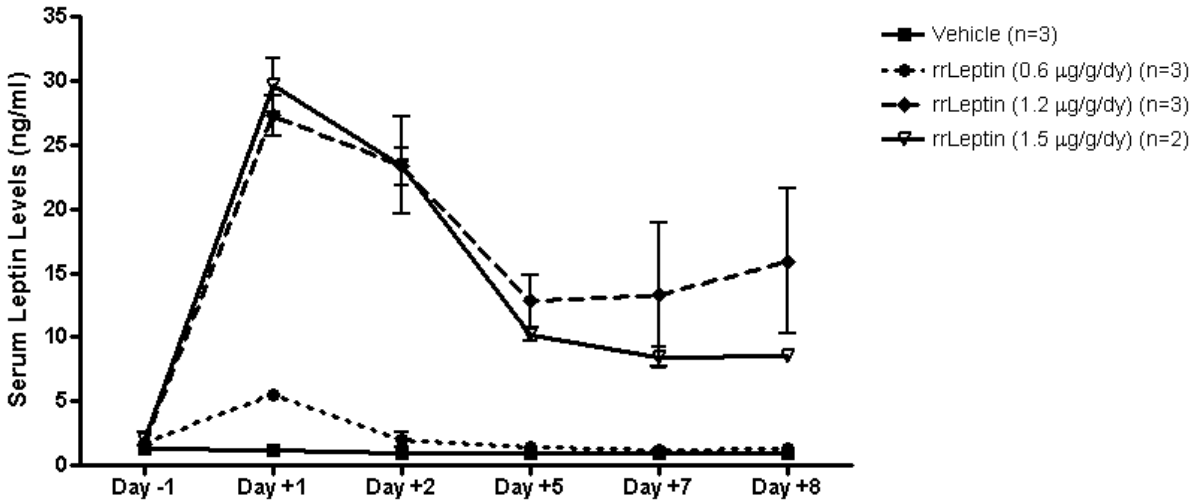


Figure 16. Serum Leptin levels from male rats treated with a single alzet pump delivering rrLeptin at the following doses for 7 days: 0.6µg/g/day, 1.2 µg/g/day or 1.5 µg/g/day. Serum leptin levels were tested by RIA, all samples were run in duplicate and mean values calculated for each sample. Serum leptin levels for rats dosed with 1.2 µg/g/day or 1.5 µg/g/day were significantly higher on all days tested ($p < 0.05$ by ANOVA comparison of all groups) than rats dosed with 0.6 µg/g/day or PBS.

Figure 17. Rats Treated with the Intermediate Dose of rrLeptin Experienced the Least Amount of Weight Gain

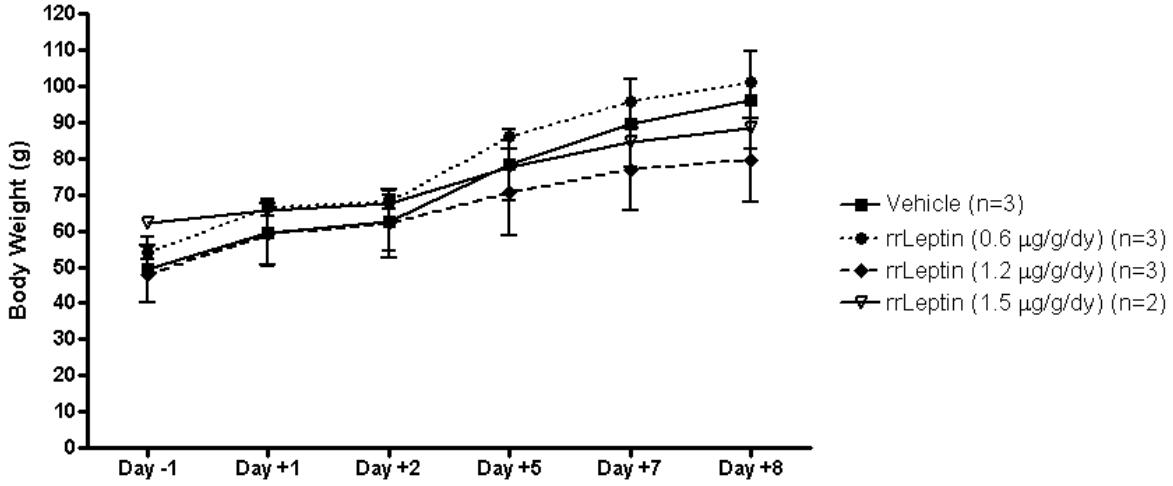


Figure 17. Rats in each treatment group were weighed daily following the insertion of pumps. Rats treated with 1.2µg/g/day of rrLeptin exhibited the least amount of gain in body weight.

Figure 18. Blood Glucose Levels Are Lowest Among Rats Treated with an Intermediate Dose of rrLeptin

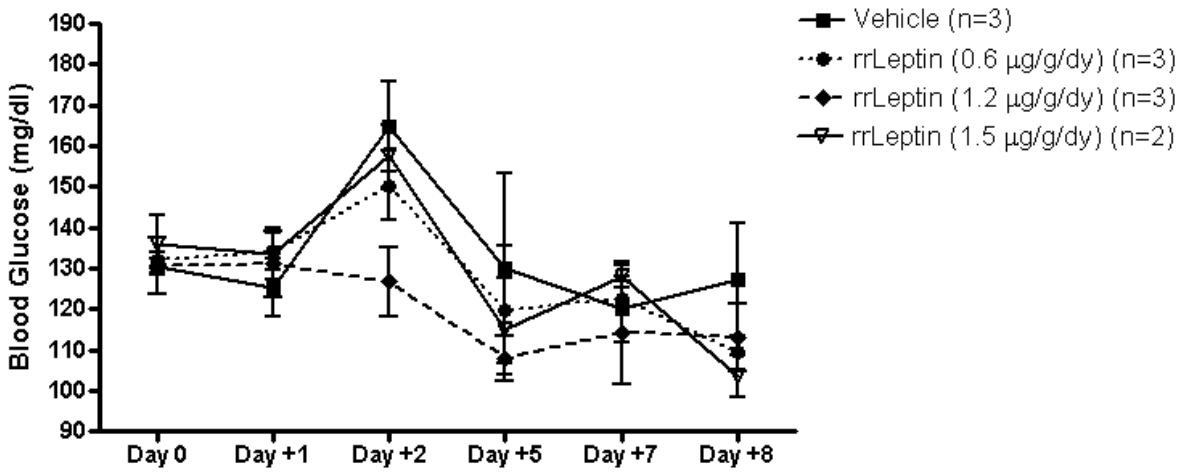


Figure 18. Non-fasting blood glucose levels were recorded daily for rats in each of the treatment groups. Rats treated with 1.2µg/g/day of rrLeptin demonstrated the lowest normal blood glucose levels on each of the follow-up days.

3. rrLeptin Protects BBDR Rats from Virally Induced T1D as Effectively as AdLeptin

Based on our pharmacokinetics study, we hypothesized that weanling rats treated with double alzet pumps, delivering rrLeptin at a dose of 1.2 µg/g/pump/day, every five days for up to 30 days would achieve the necessary serum leptin levels to match the adenovirus vector delivery system, and protect rats from developing T1D as in our pIC + KRV induction model. A schematic of our rrLeptin protection trial is depicted in **Figure 19.** Twenty one to twenty five day old female BBDR rats received either double vehicle or rrLeptin pumps six hours prior to the initiation of pIC on day -3. Only female rats were included in this study since the lack of weight gain afforded by leptin was most obvious in female rats (as observed with AdLeptin treatment). For the next two consecutive days pIC was injected i.p. and on the fourth day KRV was administered i.p. Double vehicle and rrLeptin pumps were replaced every 5 days following their initial insertion, for up to 26 days after KRV treatment to ensure that peak serum leptin levels were maintained for the period of time that the rats were most susceptible to diabetes induction. Double pumps were removed on day 29 following KRV treatment.

One hundred percent of rats (n=8) treated with rrLeptin were protected from pIC+KRV compared with 20% (1 of 5) in the vehicle control group (**Figure 20**). This survival difference was highly significant (p=0.002). rrLeptin treated female rats failed to gain weight similar to the females pretreated with AdLeptin (**Figure 21**). rrLeptin treated females weighed 85% of vehicle control rats by day 4 post KRV, and by day 20 were at 60% of the body weight of vehicle treated controls. Rats that failed to gain weight were periodically tested for urine ketones to ensure they were not undergoing starvation induced ketosis.

However, none of the rats became urine ketone positive during the study period. Following pump removals on day 29, body weights in the rrLeptin treated group rose promptly, matching the body weights of vehicle treated rats by the end of the study period. Serum leptin levels in rrLeptin treated rats peaked at 51.5 ng/ml by day 2 following KRV treatment (4 days following the first pump insertions), and remained elevated for the duration of the treatment period until pumps were removed on day 29 post KRV (**Figure 22**).

Figure 19. Schematic of the Diabetes Protection Protocol using rrLeptin Delivered by Alzet Pumps

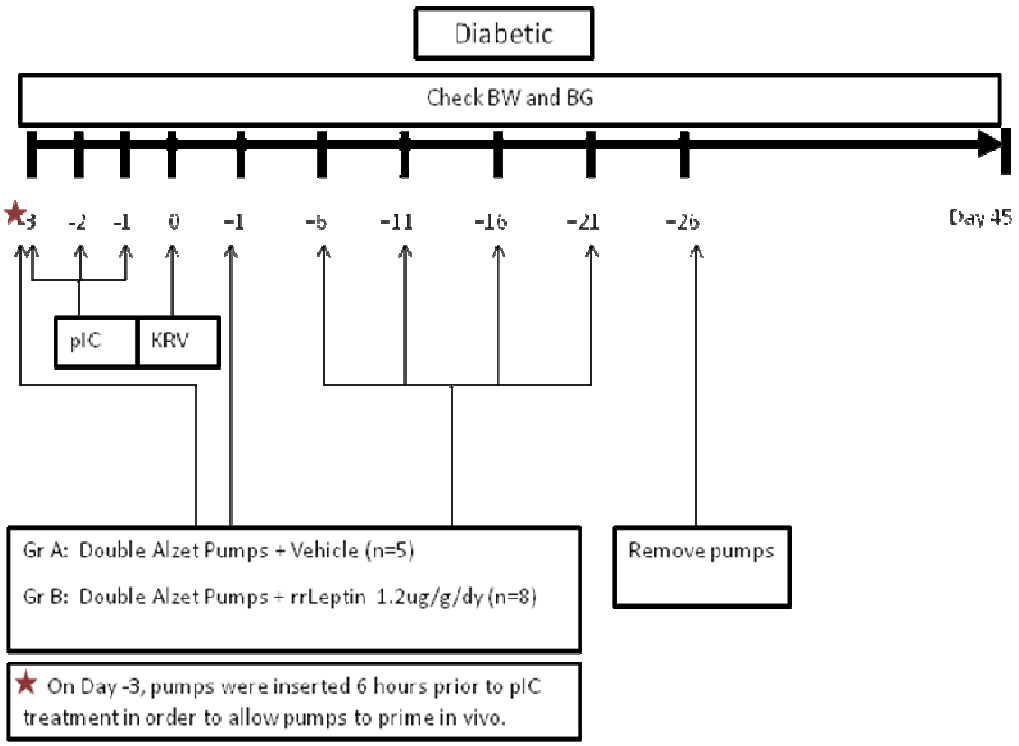


Figure 20. rrLeptin Protects 100% of BBDR Rats from pIC + KRV Induced Diabetes

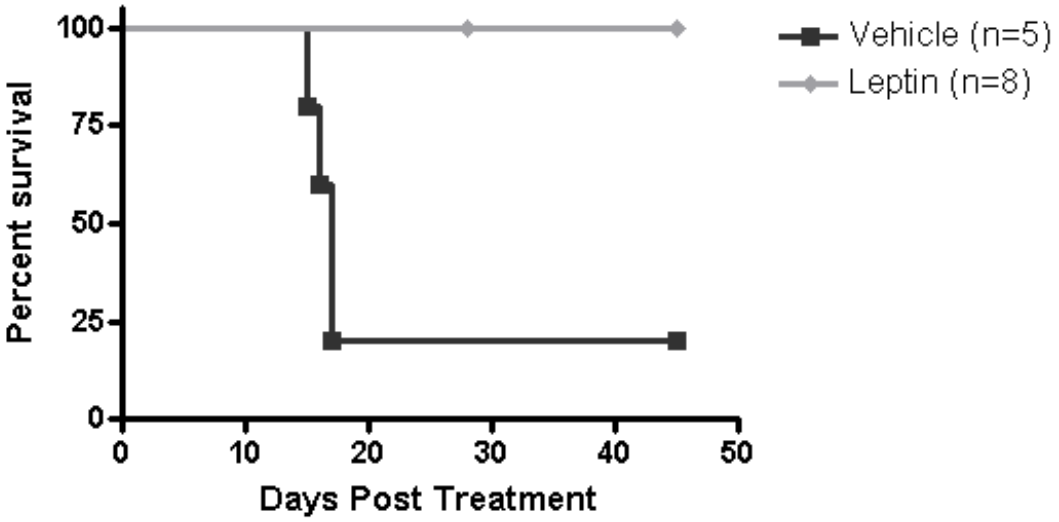


Figure 20. Twenty one to 24 day old female BBDR rats were treated with vehicle only (n=5) or rrLeptin (n=8) delivered using double alzet pumps replaced every five days for up to 29 days. Data are representative of two independent experiments. A Kaplan Meier survival curve depicts the percent of rats exhibiting diabetes free survival in each group. One hundred percent of rrLeptin treated rats compared with 20% of vehicle control treated rats were protected from type 1 diabetes (p=0.002). Median survival in the vehicle control group was 17 days.

Figure 21. rrLeptin Treated Rats Fail to Gain Weight During the Treatment Period

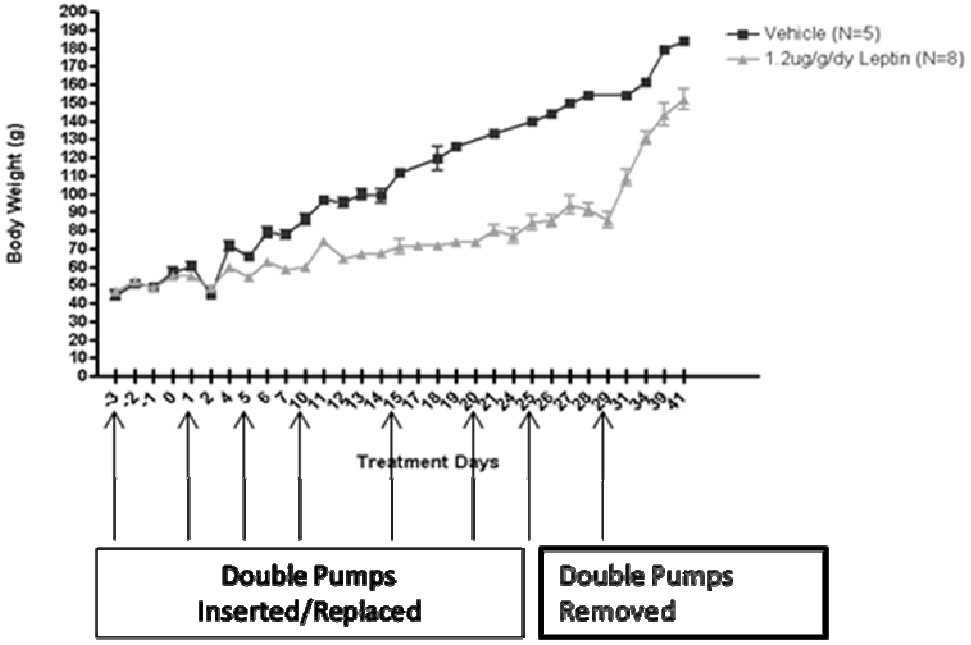


Figure 21. Body weights from female rats in each group were collected 3-5 times per week during the follow-up period. rrLeptin treated females weighed 85% of vehicle control by day 4 post KRV, and by day 20 were at 60% of the body weight of vehicle treated controls.

Figure 22. rrLeptin Delivered by Alzet Pumps Generates Serum Hyperleptinemia Comparable to AdLeptin Treated Rats

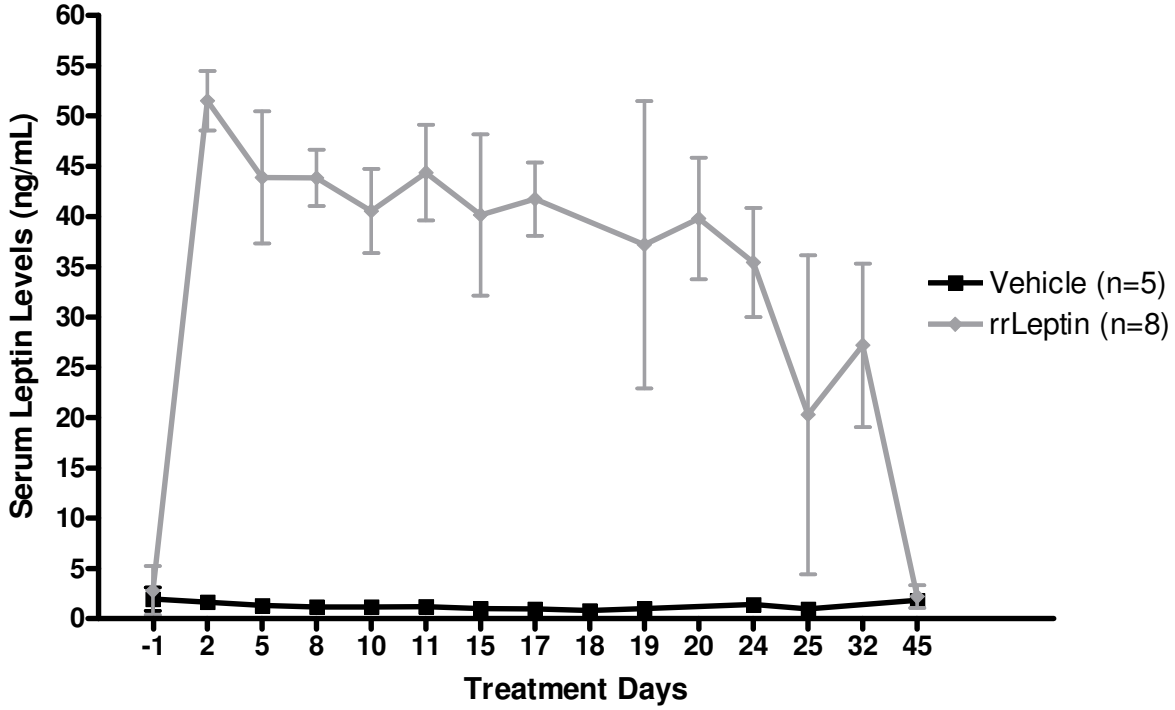


Figure 22. Serum leptin levels in rrLeptin or vehicle treated controls were measured on indicated days by RIA. Each sample was tested in duplicate and mean values for each sample was calculated. Data represent the mean serum leptin levels and the standard deviation for each group at each time point.

Figure 23. Islets from AdLeptin + pIC + KRV Protected Rats Are Protected from Insulitis and Stain Positively for Insulin

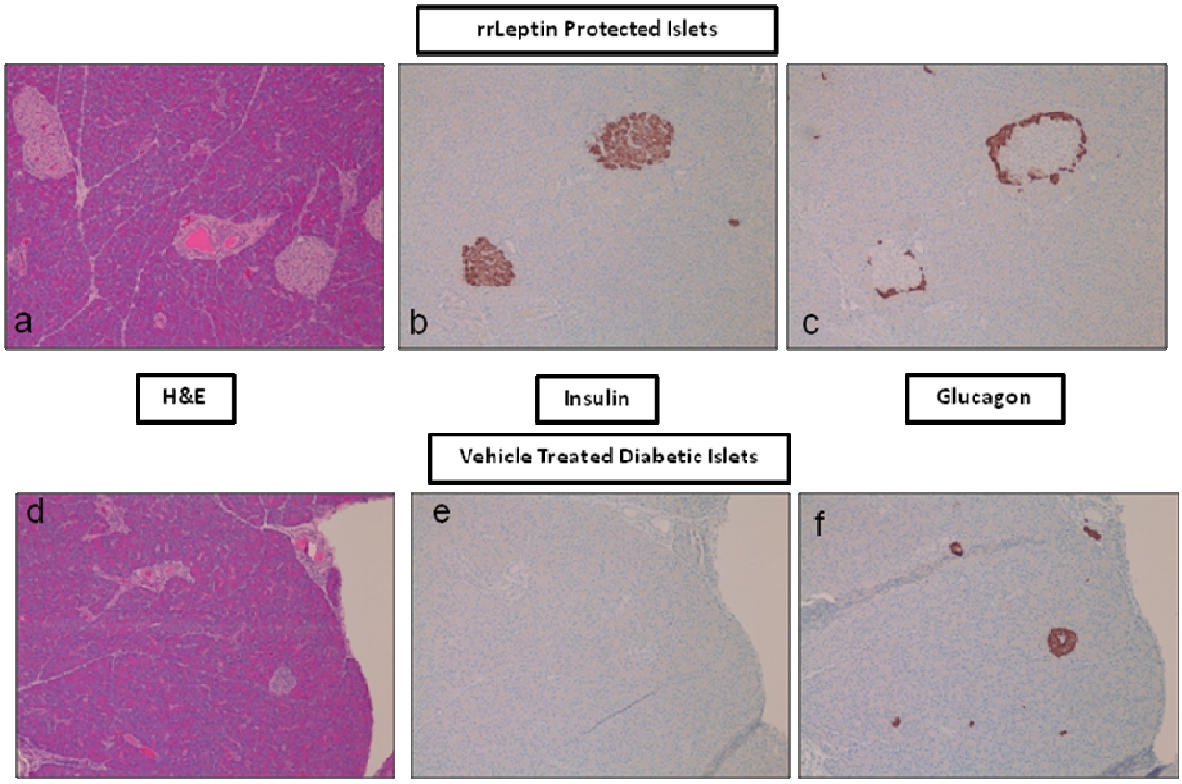


Figure 23. Upper panels: representative pancreas sections from rrLeptin + pIC + KRV treated rat protected from diabetes on day 45 (a) H&E of pancreas demonstrates minimal to no insulitic infiltrate in or around islets (b) insulin staining by IHC demonstrates positive central staining of beta cells producing insulin and (c) glucagon staining by IHC demonstrates strong peripheral staining of alpha cells. Lower panels: representative pancreas sections from day 45 of a diabetic rat treated with vehicle + pIC + KRV. (d) H&E demonstrates involuted, shrunken islets and no remnant insulitis (e) no insulin staining by IHC due to complete beta cell destruction and (f) central glucagon staining from alpha cells of involuted islets. All images obtained at 10x magnification.

In this study we were able to demonstrate, first, that adenovirus-free delivery of leptin using alzet pumps could generate a hyperleptinemic state comparable with that generated with AdLeptin, and, second, that rrLeptin was as effective in protecting rats from autoimmune diabetes as was AdLeptin. In fact, none of the rats treated with rrLeptin developed diabetes during the entire study period, whereas two rats in the AdLeptin treated cohort developed diabetes despite elevated serum leptin levels. Islet histology also confirmed that the islets of rrLeptin treated rats were free of insulitis and stained positive for insulin and glucagon by IHC. In contrast, islets from diabetic vehicle treated rats appeared as end-stage islets, with no insulin staining, and central glucagon staining resulting from the absence of beta cells in the islets (**Figure 23**). Furthermore, there was no remnant insulitis in these islets presumably due to complete beta cell destruction and subsequent departure of beta cell-reactive immune cells.

Summary

The adipokine, leptin, has been demonstrated to inhibit glucose stimulated insulin secretion from beta cells and protect beta cells from lipoapoptosis [98, 99]. We hypothesized that a hyperleptinemic state might afford a similar protection for beta cells in an autoimmune model of T1D. To test this hypothesis, we pretreated weanling BBDR rats with AdLeptin or a control adenovirus vector, AdBetagal, or PBS i.v. prior to initiating our diabetes induction protocol with pIC + KRV. Ninety one percent of AdLeptin pretreated rats were protected from T1D, compared with 50% of AdBetagal and 10% of the PBS pretreated rats. Diabetes free survival in the AdLeptin treated rats was associated with induction of a hyperleptinemic state with serum leptin levels peaking at 35 ng/ml on the day of KRV treatment, and

declining subsequently. Hyperleptinemia prevented appropriate weight gain in both genders, with treated animals weighing 65% of their control counterparts by day 14 post KRV. Non-fasting serum insulin levels in AdLeptin treated rats was also significantly lower on days 0 and 14 of follow-up, consistent with the known effects of leptin to suppress insulin secretion from beta cells. Islets from AdLeptin treated non-diabetic rats exhibited minimal to no insulitis and stained positively for insulin and glucagon at the end of the study.

Because 50% of AdBetagal treated rats were also protected from T1D, we chose to test a virus free leptin delivery system so as to eliminate any effects the adenovirus may have in protecting rats from pIC + KRV induced diabetes. We performed a dose response study of rrLeptin in male BBDR rats and determined that 1.2µg/g/day per pump using double pumps would achieve serum leptin levels similar to AdLeptin treatment. We then tested the effect of pump delivered rrLeptin in our pIC + KRV model. Female BBDR rats were treated with double pumps containing either vehicle control or rrLeptin followed by pIC + KRV. Double pumps in both groups were replaced every five days for up to 26 days post KRV, followed by their removal on day 29. Adapting the use of rrleptin in an adenovirus free delivery system, we observed that 100% of rrLeptin treated rats were protected from T1D, compared with 20% of vehicle control treated rats. This significant survival advantage was associated with the generation of a hyperleptinemic state where serum leptin levels peaked at 51.5 ng/ml as quickly as two days following pump insertion. rrLeptin treated rats weighed significantly less than their vehicle control treated counterparts, an effect that quickly dissipated once the pumps were removed on day 29. In this study we demonstrated that adenovirus free delivery of leptin in BBDR rats can protect them from virally induced T1D. Further studies will

investigate the mechanisms by which the hyperleptinemic state created by either AdLeptin or rrLeptin protects rats from autoimmune T1D.

CHAPTER V: IMMUNE MECHANISMS OF LEPTIN MEDIATED PREVENTION FROM pIC + KRV INDUCED TYPE 1 DIABETES

Introduction

We have demonstrated high serum leptin levels (35 – 50 ng/ml) achieved by both an adenovirus and alzet pump delivery system, could prevent pIC + KRV induced T1D in weanling BBDR rats. This hyperleptinemic state was able to protect islets from autoreactive beta cell destruction by preventing insulitis, the accumulation of lymphocytes around the islets. We also showed that hyperleptinemia was associated with lower non-fasting serum insulin levels in AdLeptin treated rats. The lack of insulitis surrounding the islets of leptinized rats led us to surmise that leptin might relegate the beta cell into a state of relative quiescence whereby beta cells are ignored by the immune system. We hypothesize that the protection against autoimmunity afforded by leptin is from its combined effects on the beta cell and immune cells. Leptin has been primarily demonstrated to have proinflammatory and proautoimmune effects on the immune system of both humans and rodents [59, 76]. However some studies have shown that it could inhibit lymphocyte proliferation if delivered directly into the CNS or if used in lymphocyte cultures at high doses [100, 101]. Due to it observed prevention of T1D in our pIC + KRV model, we wanted to elucidate the effects of the hyperleptinemic state on the immune system in this model.

Results

1. *rrLeptin Treatment Decreases Spleen Weights and Total Splenic Cellularity*

To dissect the mechanism of rrLeptin mediated protection from pIC + KRV- induced T1D, we evaluated changes in lymphocyte populations in the spleen and pancreatic draining lymph node (PLN) during the early phases of virus induction. Previous studies have shown that activated lymphocytes can be detected in the PLN and spleens as early as day 4-6 following KRV treatment, prior to diabetes onset [52]. To assess if hyperleptinemia altered these responses at an early time point of diabetes induction, we performed three independent experiments. A schematic of this experimental design is depicted in **Figure 24**.

In each experiment, we inserted double alzet pumps (day -3) which delivered either vehicle control or 1.2 µg/g/day of rrLeptin into 21-25 day old female BBDR rats. Six hours later, all rats were given 1 µg/g body weight of pIC i.p. Rats were treated for two more consecutive days with i.p. pIC followed by a single dose of 10^7 pfu of KRV i.p. (day 0). In one experiment a third group was added to the protocol (rats treated with vehicle pumps alone without pIC or KRV treatment) to track baseline changes in various immune populations within this model. In every experiment, pumps were replaced in all treatment groups on day 1 (post-KRV). Body weights and non-fasting blood glucose levels were monitored daily for each group in all experiments. On the sixth day following KRV treatment, rats were sacrificed and their spleens, cervical lymph nodes (CLN) and PLN were harvested for flow cytometery analysis. CLNs served as control lymph nodes for the PLNs. In one experiment we also collected spleen weights.

By day 6 following KRV, rrLeptin treated rats (LPK) from each of the three experiments weighed 64%, 74% and 75% of the body weights of vehicle control treated rats (VPK) (**Figure 25**). Upon sacrifice and organ harvest in each experiment, we noticed an obvious decrease in the spleen size of the LPK rats compared with VPK treated rats. To quantify this, we measured the gross spleen weights and spleen to body weight ratio in one experiment. We observed a 60% decrease in the gross spleen weight, and a 47% decrease in the spleen to body weight ratio in the LPK rats compared with VPK treated rats ($p=0.006$ and $p=0.002$, respectively, **Figure 26a, 26b**). The decrease in the spleen to body weight ratio in the LPK treated rats indicated that perhaps rrLeptin treatment was further reducing spleen weights beyond the expected decrease associated with low body weight. Additionally, total splenic cell counts were significantly lower in the rrLeptin treated group compared with other groups in all three experiments (**Figure 27**).

Figure 24. Schematic Diagram of the Protocol to Evaluate the Effects of rrLeptin on the Immune System

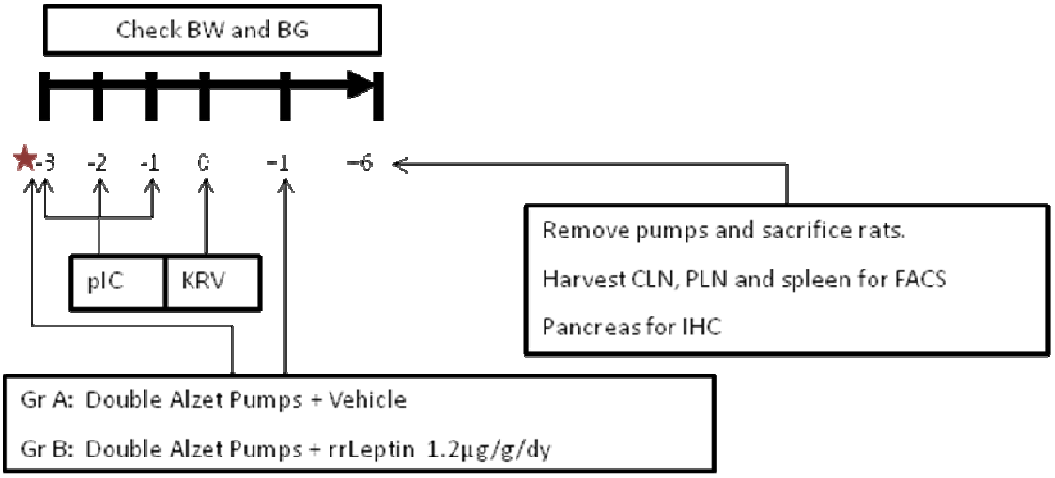


Figure 25. rrLeptin Prevents Appropriate Weight Gain in Female BBDR Rats

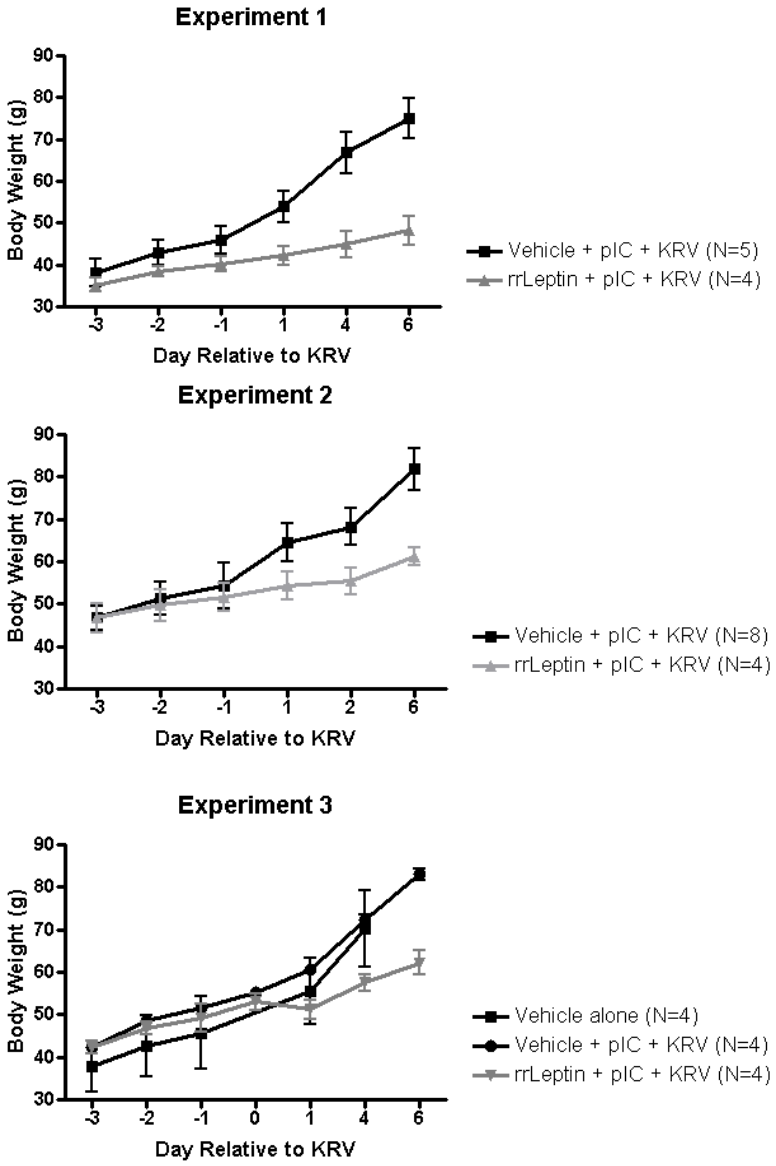


Figure 25. Body weights from female BBDR rats undergoing pIC + KRV induction and treated with double alzet pumps delivering vehicle or rrLeptin. Data from three independent experiments are shown. Experiment 3 has an additional group, vehicle alone, that did not undergo pIC + KRV induction.

Figure 26. rrLeptin Precipitates a Decline in Spleen Weight and Spleen to Body Weight Ratio

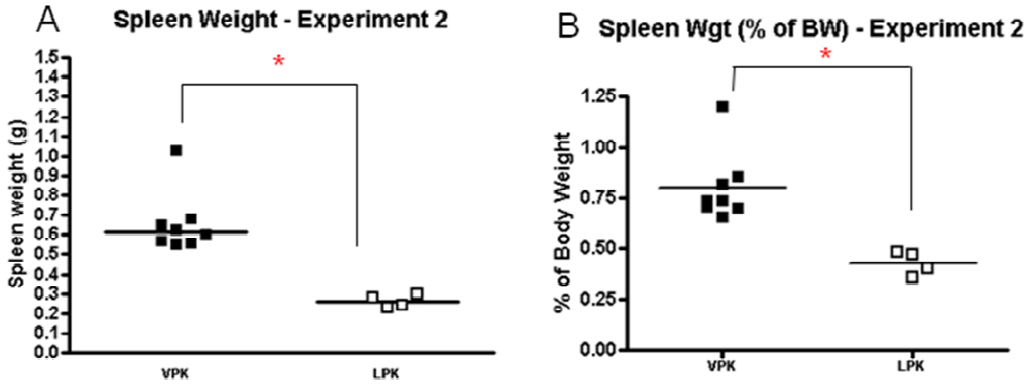


Figure 26. (A) Gross spleen weights and (B) spleen to body weight ratio of vehicle + pIC + KRV (VPK) and rrLeptin + pIC + KRV (LPK) treated rats obtained at the time of sacrifice on day 6. Spleen to body weight ratio calculated based on the spleen and body weights obtained at the time of sacrifice on day 6. Statistical comparisons between the VPK and LPK groups are based on Student's T test (*p=0.0006 for A, and *p=0.002 for B).

Figure 27. rrLeptin Treatment Lowers Total Spleen Counts

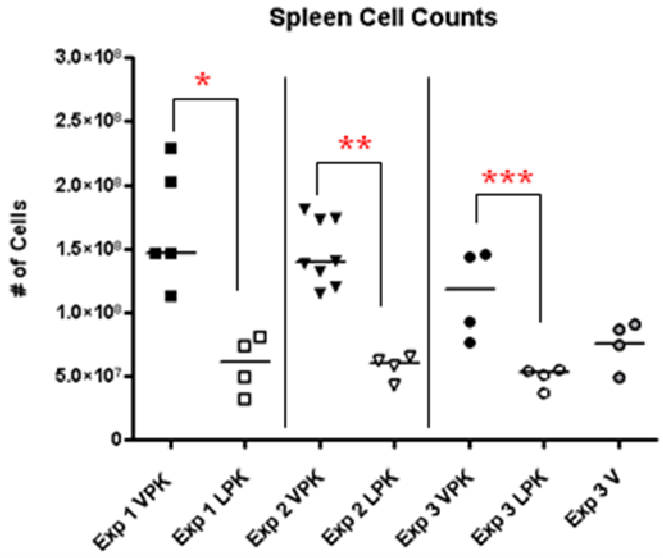


Figure 27. Total spleen counts from vehicle + pIC + KRV (VPK) and rrLeptin + pIC + KRV (LPK) treated BBDR rats were measured in 3 independent experiments. Experiment 3 additionally included total spleen counts from BBDR rats treated with vehicle alone (V). Total spleen counts are Coulter counts based on single cell suspensions of the homogenized spleens of rats in each treatment group. Statistical comparisons between the VPK and LPK groups are based on Student's T test for experiments 1 and 2 (*p = 0.004, **p=0.001), and ANOVA followed by Bonferroni comparison of groups for experiment 3 (***p<0.05).

2. rrLeptin Decreases Total Splenic Lymphocyte and APC Cell Numbers

To determine if decreased splenic cellularity was associated with a reduction in specific splenic subpopulations, we quantified lymphocyte and antigen presenting cell (APC) subsets in the spleen. For each experiment, we analyzed lymphocyte and APC subset frequencies by flow cytometry, as well as total numbers of these subsets in the spleen. Data on splenic lymphocyte populations pooled from three experiments revealed that there was a small decrease (5%, $p<0.05$) in the fraction of live cells in the vehicle + pIC + KRV treated group (VPK) compared with the rrLeptin + pIC + KRV group (LPK) (**Figure 28**). All subsequent cell population frequencies were gated from the live population, so that only the fraction of live cell subsets is reported from this point forward. We found a 4% increase in the overall frequency of TCR+ cells in LPK versus VPK splenocytes ($p<0.05$) however, the frequency of CD4+ and CD8+ cells did not change significantly in between any of the treatment groups (**Figure 28**). The frequency of splenic dendritic cell and macrophages (DC/MACs) were not also significantly different between treatment groups, however, the frequency of splenic B cells was 3.5% higher in the LPK than the VPK group ($p<0.05$) (**Figure 29**).

Figure 28. rrLeptin Increases the Proportion of Splenic TCR+ Cells

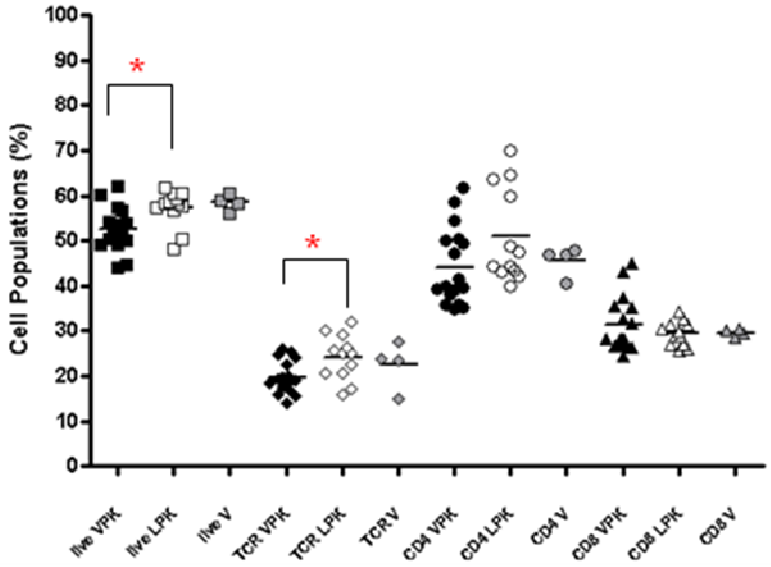


Figure 28. Percentage of live, TCR+, CD4+ and CD8+ T cells from the spleens of vehicle + pIC + KRV (VPK - black symbols), rrLeptin + pIC + KRV (LPK - clear symbols) and vehicle only (V - grey symbols) treated rats on day 6 post KRV treatment.. TCR+ cells were gated from the live lymphocyte population (live/dead blue negative). CD4+ and CD8+ cells were gated from all TCR+ cells. Data pooled from 3 independent experiments. Statistical analysis included ANOVA followed by Bonferroni comparison of each group pair (*$p<0.05$).

Figure 29. rrLeptin Increases Proportion of Splenic B Cells

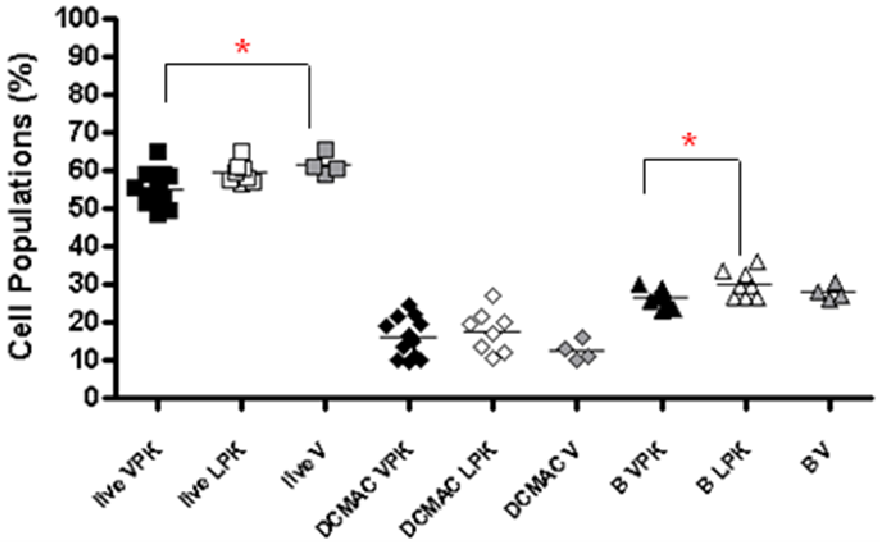


Figure 29. Percentage of live, DCs and macrophages (CD11b/c+), and B cells (CD45RA +) cells from the spleens of vehicle + pIC + KRV (VPK - black symbols), rrLeptin + pIC + KRV (LPK - white symbols) and vehicle only (V - grey symbols) treated rats on day 6 post KRV treatment. DC/MAC and B cell populations were gated from the live population (live/dead blue negative). Data pooled from 3 independent experiments. Statistical analysis included ANOVA followed by Bonferroni comparison of each group pair (*$p<0.05$).

Figure 30. rrLeptin Decreases the Total Number of Splenic TCR+ CD4+ and CD8+ T Cells

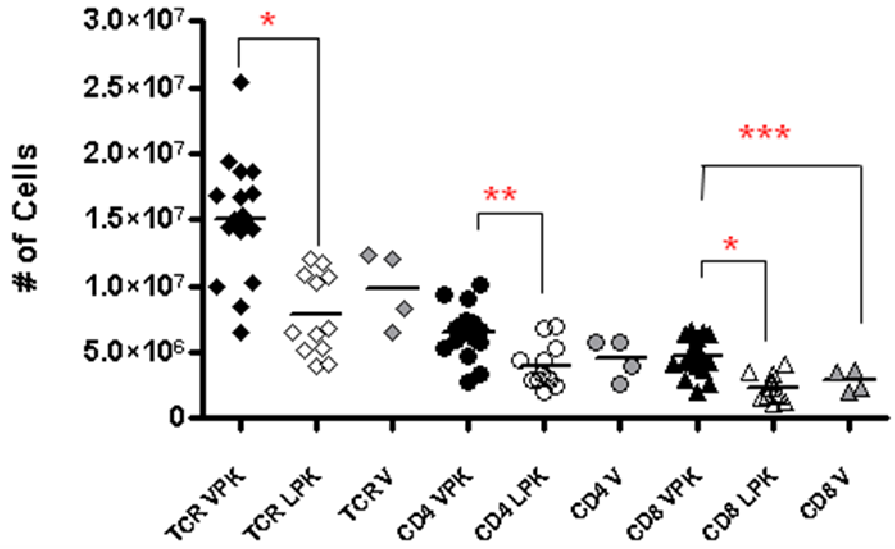


Figure 30. Total number of live TCR+, CD4+ and CD8+ T cells from the spleens of vehicle + pIC + KRV (VPK - black symbols), rrLeptin + pIC + KRV (LPK - white symbols) and vehicle only (V - grey symbols) treated rats on day 6 post KRV treatment. Statistical analysis included ANOVA followed by Bonferroni comparison of each group pair (*$p<0.001$, **$p<0.01$, ***$p<0.05$).

Figure 31. rrLeptin Decreases the Total Number of Splenic DC/MACs and B Cells

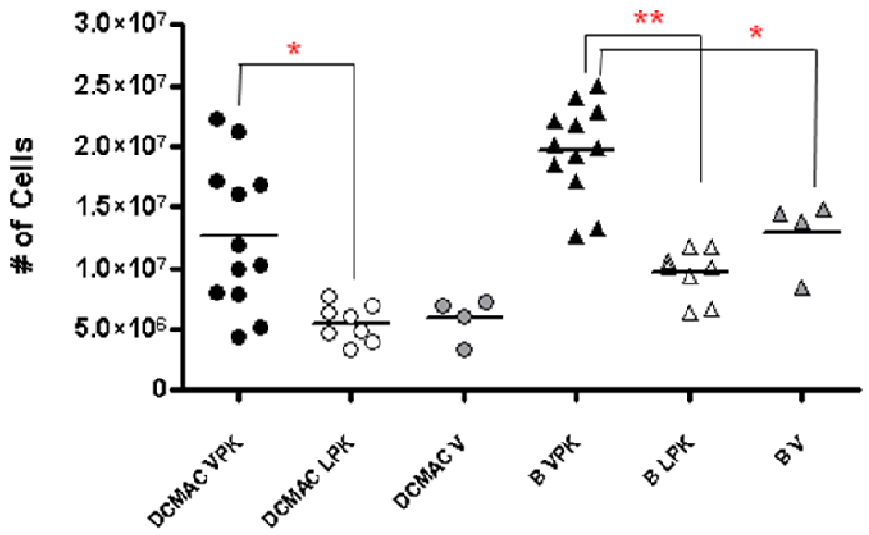


Figure 31. Total number of live DC/MACS (CD11b/c+) and B cells (CD45RA+) from the spleens of vehicle + pIC + KRV (VPK - black symbols), rrLeptin + pIC + KRV (LPK - white symbols) and vehicle only (V - grey symbols) treated rats on day 6 post KRV treatment. Data pooled from two independent experiments. Statistical analysis included ANOVA followed by Bonferroni comparison of each group pair (*p <0.01, **p<0.001).

When taking into consideration the decreased cell counts of spleens from rrLeptin treated rats, and applying the population proportions to the total cell counts, we observed significant differences in the total number of cells in various subsets. Total numbers of TCR+, CD4+, CD8+, DC/MACs and B cells were between 1.6 – 2.4 times lower in the LPK treated rats compared with VPK treated rats, and not significantly different from those in the vehicle alone group (**Figures 30 and 31**).

Subsequently, we wanted to determine the frequencies and total numbers of selected T cell and APC subsets. Among T cell subsets, we examined rrLeptin induced changes in activated CD4+ or CD8+ T cells, or Tregs (CD4+CD25+Foxp3+). Although we did not observe any differences in the proportions of activated CD4+ T cells (CD4+CD25+) between the VPK, LPK and vehicle only groups, there was a significant increase in the proportion of activated CD8+ T cells (CD8+CD25+) in both the groups treated with pIC + KRV (VPK and LPK) relative to vehicle only controls (**Figure 32**). We attribute this increase in activated CD8+CD25+ to the viral immune response in the VPK and LPK groups, and conclude that rrLeptin treatment did not affect the change in activation of CD8+ cells in response to virus in our model. We were only able to compare the frequencies of Tregs in the LPK and VPK groups, as there were too few of these cells in the vehicle only group to provide reliable cell frequencies. There was no significant difference in the frequency of Tregs between VPK and LPK treated rats. Additionally, the total number of activated CD4+CD25+ and Treg populations was not significantly different between any groups. LPK treated rats, however, had significantly lower CD8+CD25+ cells in their spleen than VPK treated rats (**Figures 32 and 33**).

Within APC subsets, we looked for differences in the proportion and number of DC/MACs and B cells expressing class II MHC and the co-stimulatory molecule CD86. There were no significant differences in the percentages of all DC/MACs or B cells expressing class II or the subsets co-expressing class II MHC and CD86 between treatment groups (**Figures 34 and 35**). When we analyzed the differences in the total numbers of APC subsets, we observed a significant decrease (1.9 – 2.5 fold) in both the total number of DC/MACs and B cells expressing class II MHC in the LPK versus VPK treatment groups (**Figures 36 and 37**). There was also a significant decrease (2.4 – 2.5 fold) in the total number of B cells co-expressing class II MHC and CD86 in the LPK versus VPK group (**Figure 37**), but a similar statistically significant decrease in the number of DC/MACs co-expressing class II MHC and CD86 in the LPK group was not observed (**Figure 36**).

3. rrLeptin Does Not Change Lymph Node Populations

There were no statistically significant changes in the proportions of TCR+, CD4+, CD8+, DC/MACs or B cells in the CLN or PLN from vehicle or rrLeptin treated rats undergoing pIC + KRV induction (**Figures 38 and 39**). There were also no statistically significant differences in any subsets of CD4+, CD8+, DC/MACs or B cells (**Figures 40 and 41**) in either tissue between treatment groups. Due to the variation in the number of lymph nodes harvested in each experiment, we chose not to analyze differences in the total numbers of each cell type within each treatment group.

Figure 32. rrLeptin Does Not Change the Proportions of Various Splenic T Cell Subsets

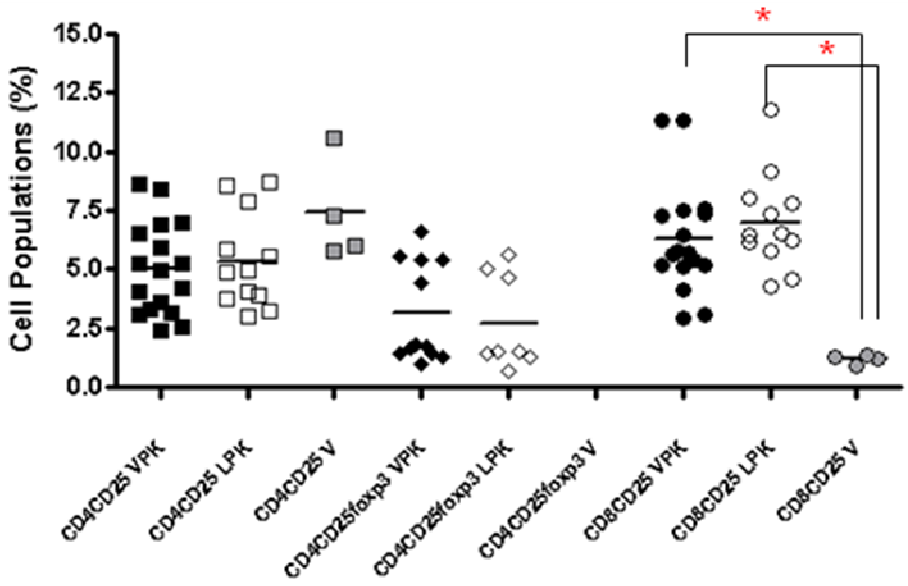


Figure 32. Percentage of live CD4+ and CD8+ T cell subsets from the spleens of vehicle + pIC + KRV (VPK - black symbols), rrLeptin + pIC + KRV (LPK - white symbols) and vehicle only (V -grey symbols) treated rats on day 6 post KRV treatment. Subsets of CD4+ and CD8+ cells were gated following selection of the CD4+ or CD8+ cells. No percentages were calculated for the CD4+CD25+foxp3+ population in the vehicle only group due to small cell counts in that subset. Data pooled from 3 independent experiments. Statistical analysis included ANOVA followed by Bonferroni comparison of each group pair (*$p<0.001$).

Figure 33. rrLeptin Decreases the Total Numbers of Splenic CD8+CD25+ Cell Subsets

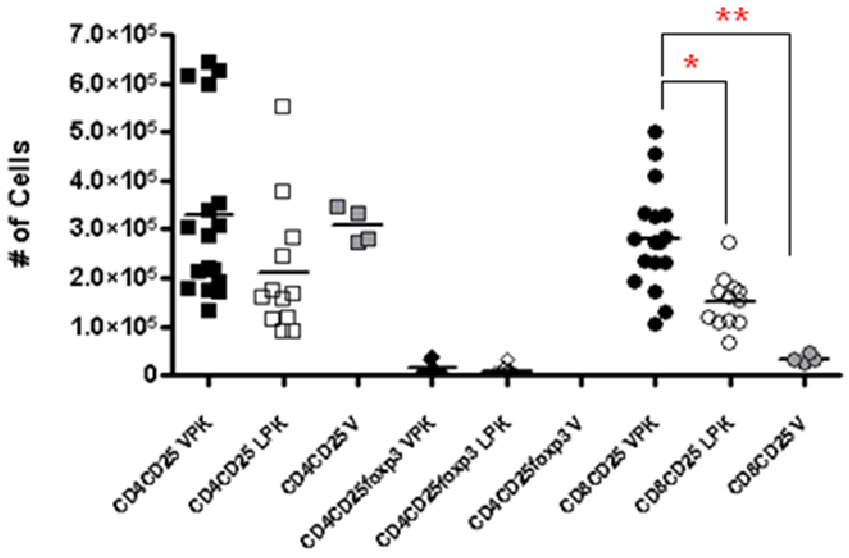


Figure 33. Total number of live CD4+ and CD8+ T cell subsets from the spleens of vehicle + pIC + KRV (VPK - black symbols), rrLeptin + pIC + KRV (LPK - white symbols) and vehicle only (V - grey symbols) treated rats on day 6 post KRV treatment. Data pooled from 3 independent experiments. Statistical analysis included ANOVA followed by Bonferroni comparison of each group pair (*$p<0.01$, **$p<0.001$).

Figure 34. rrLeptin Does Not Change the Frequency of Splenic DC/MACs Expressing Class II MHC Alone or Class II with the Costimulatory Molecule, CD86

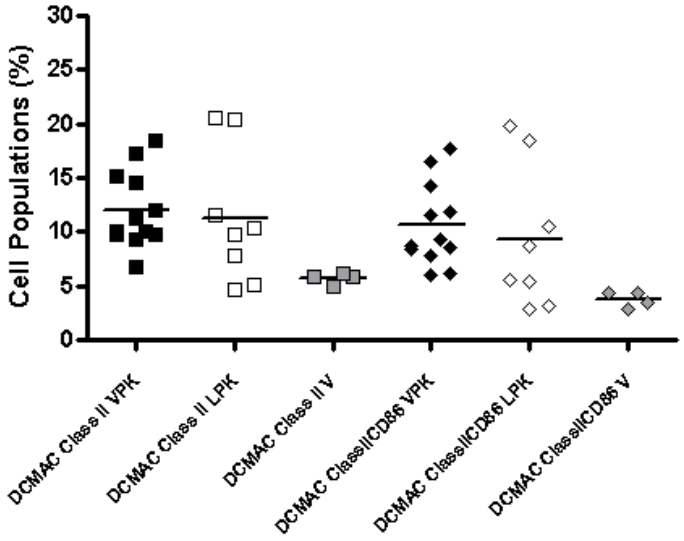


Figure 34. Percentage of live DC/MACs subsets from the spleens of vehicle + pIC + KRV (VPK - black symbols), rrLeptin + pIC + KRV (LPK - white symbols) and vehicle only (V - grey symbols) treated rats on day 6 post KRV treatment. DC/MAC Class II + cells (RT1B+) and ClassII+CD86+ cells were gated following selection of all live DC/MAC+ cells. Data pooled from 2 independent experiments. Statistical analysis included ANOVA followed by Bonferroni comparison of each group pair (no significant differences by ANOVA).

Figure 35. rrLeptin Does Not Change the Frequency of Splenic B Cells Expressing Class II MHC or the Costimulatory Molecule, CD86

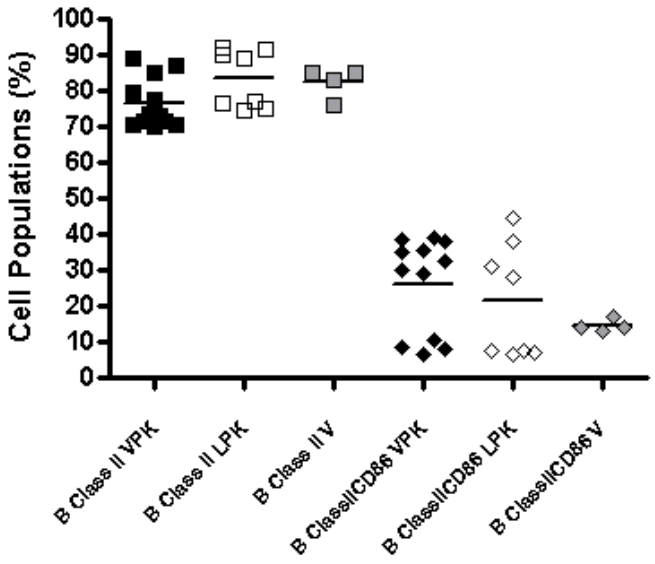


Figure 35. Percentage of live B cell subsets from the spleens of vehicle + pIC + KRV (VPK - black symbols), rrLeptin + pIC + KRV (LPK - white symbols) and vehicle only (V - grey symbols) treated rats on day 6 post KRV treatment. B Class II + cells (RT1B+) and ClassII+CD86+ cells were gated following selection of all live B cells (CD45RA+). Data pooled from 2 independent experiments. Statistical analysis included ANOVA followed by Bonferroni comparison of each group pair (no significant differences by ANOVA).

Figure 36. rrLeptin Decreases the Total Number of DC/MACs Expressing Class II MHC But Not the Number Expressing Class II MHC and the Costimulatory Molecule, CD86

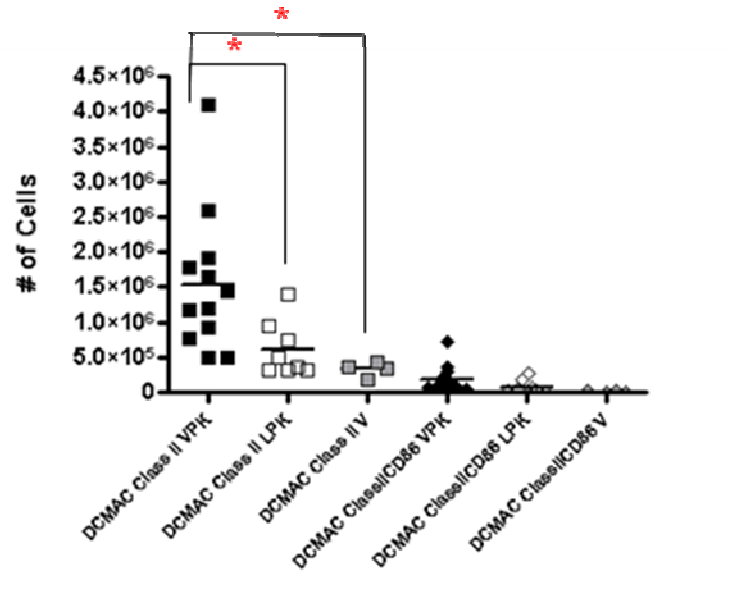


Figure 36. Total number of live DC/MACs subsets from the spleens of vehicle + pIC + KRV (VPK - black symbols), rrLeptin + pIC + KRV (LPK - white symbols) and vehicle only (V - grey symbols) treated rats on day 6 post KRV treatment. Data pooled from 2 independent experiments. Statistical analysis included ANOVA followed by Bonferroni comparison of each group pair (*$p<0.05$).

Figure 37. rrLeptin Decreases the Total Number of B Cells Expressing Class II MHC and the Costimulatory Molecule, CD86

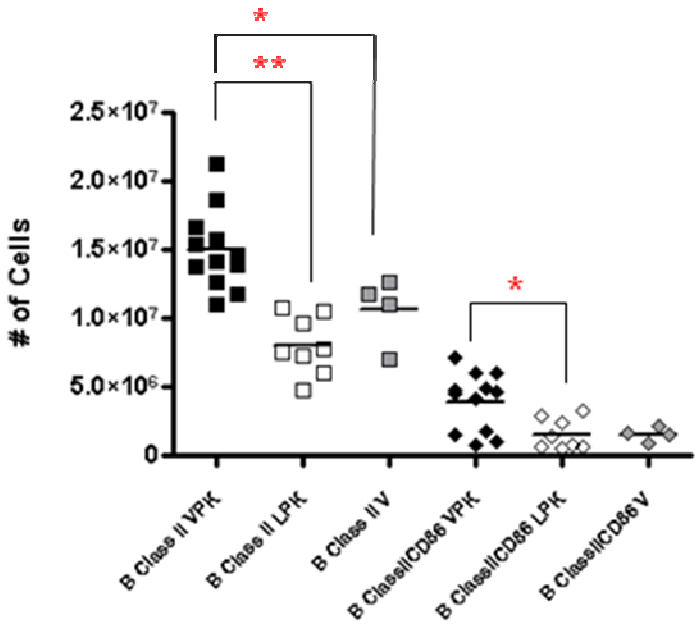


Figure 37. Total number of live B cell subsets from the spleens of vehicle + pIC + KRV (VPK - black symbols), rrLeptin + pIC + KRV (LPK - white symbols) and vehicle only (V - grey symbols) treated rats on day 6 post KRV treatment. Data pooled from 2 independent experiments. Statistical analysis included ANOVA followed by Bonferroni comparison of each group pair (*$p<0.05$, **$p<0.001$).

Figure 38. rrLeptin Does Not Alter the Frequency of T Cell Populations in Lymph Nodes of pIC + KRV Treated Rats

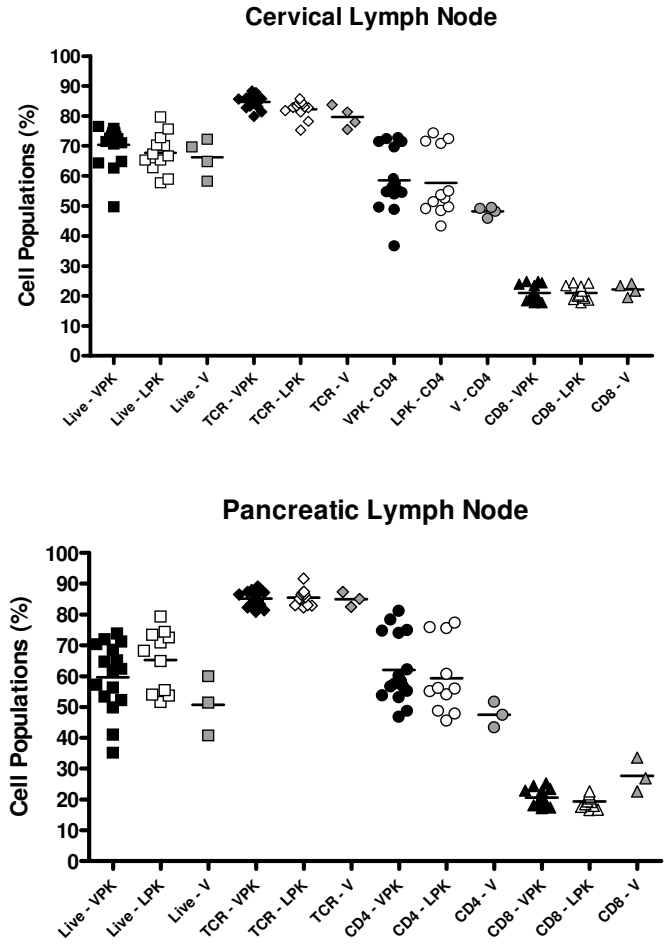


Figure 38. Percentage of live, TCR+, CD4+ and CD8+ T cells from the (A) cervical and (B) pancreatic draining lymph node of vehicle + pIC + KRV (VPK - black symbols), rrLeptin + pIC + KRV (LPK - white symbols) and vehicle only (V - grey symbols) treated rats on day 6 post KRV treatment. TCR+ cells were gated following selection of the live lymphocyte population (live/dead blue negative). CD4+ and CD8+ cells were then gated following selection of the total live TCR+ population. Data pooled from 3 independent experiments. No significant differences between groups by ANOVA comparison.

Figure 39. rrLeptin Does Not Alter the Frequencies of APCs in the Lymph Nodes of pIC + KRV Treated Rats

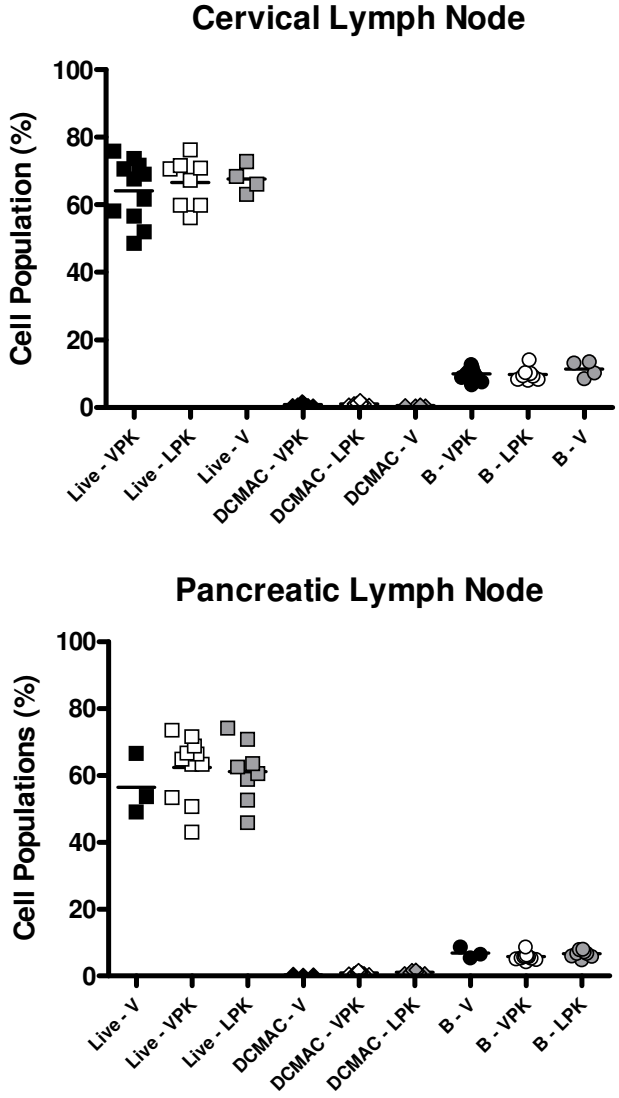


Figure 39. Percentage of live, DC s and macrophages (CD11b/c+), and B cells (CD45RA +) cells from the cervical (CLN) and pancreatic draining lymph node (PLN) of vehicle + pIC + KRV (VPK - black symbols), rrLeptin + pIC + KRV (LPK - white symbols) and vehicle only (V - grey symbols) treated rats on day 6 post KRV treatment. DC/MAC and B cell populations were gated following selection of the total live cell population (live/dead blue negative). Data pooled from 2 independent experiments. No significant differences between groups by ANOVA comparison.

Figure 40. rrLeptin Does Not Alter Frequencies of Lymph Node T Cell Subsets in pIC + KRV Treated Rats

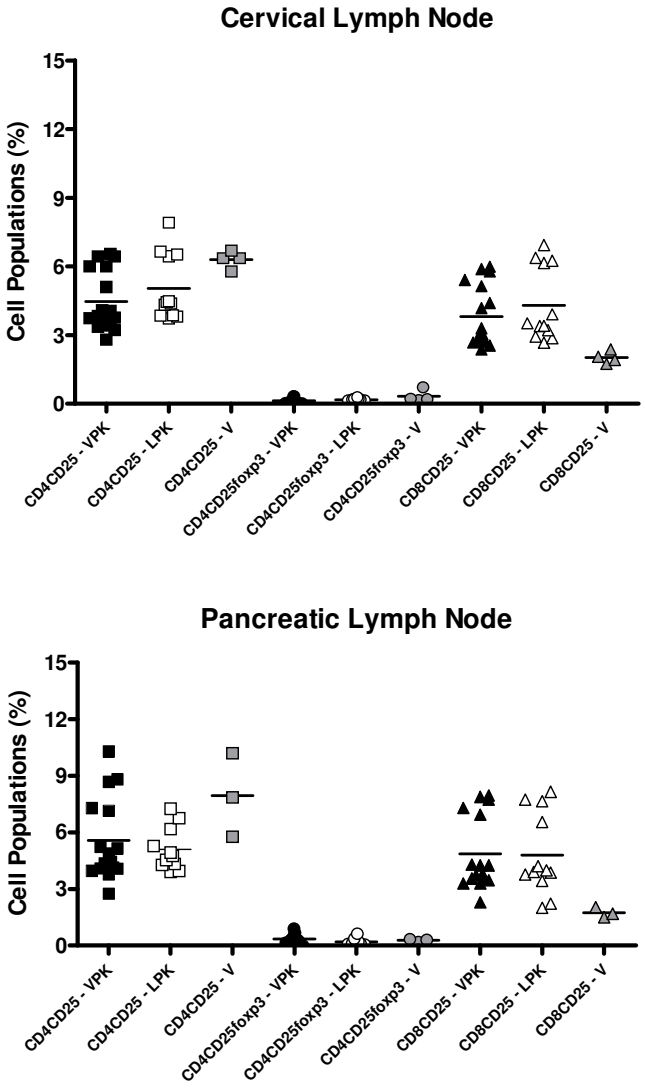


Figure 40. Percentage of live CD4+ and CD8+ T cell subsets from the spleens of vehicle + pIC + KRV (VPK - black symbols), rrLeptin + pIC + KRV (LPK - white symbols) and vehicle only (V - grey symbols) treated rats on day 6 post KRV treatment. Subsets of CD4+ and CD8+ cells were gated following selection of the CD4+ or CD8+ cells. No percentages were calculated for the CD4+CD25+foxp3+ population in the vehicle only group due to small cell counts in that subset. Data pooled from 3 independent experiments. No significant differences between groups by ANOVA comparison.

Figure 41. rrLeptin Does Not Alter Frequencies of Lymph Node APC Subsets in pIC + KRV Treated Rats

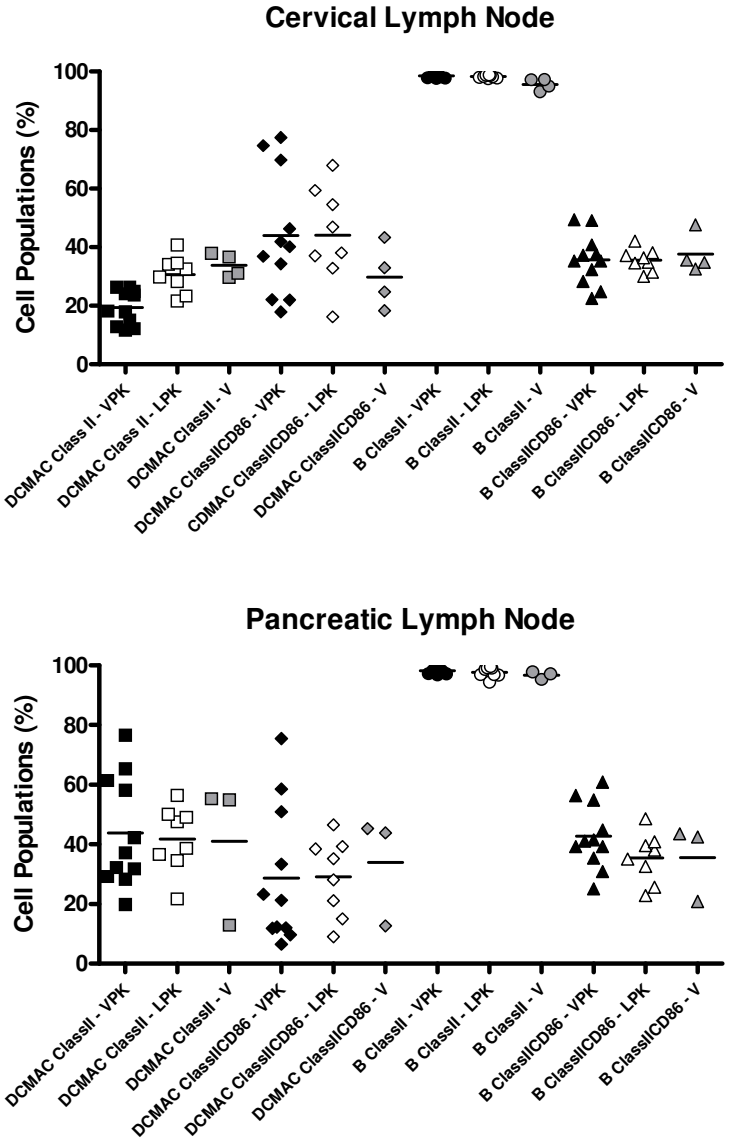


Figure 41. Percentage of live DC/MAC and B cell subsets from the spleens of vehicle + pIC + KRV (VPK - black symbols), rrLeptin + pIC + KRV (LPK - white symbols) and vehicle only (V - grey symbols) treated rats on day 6 post KRV treatment. DC/MAC and B Class II + cells (RT1B+) and ClassII+CD86+ cells were gated following selection of all live DC/MAC (CD11b/c+) and B cells (CD45RA+). Data pooled from 2 independent experiments. No significant differences between groups by ANOVA comparison.

4. Changes in the PBMC Populations with rrLeptin Treatment

To complement our spleen and lymph node data, we wanted to determine if rrLeptin induced any changes in the peripheral blood mononuclear cell (PBMC) population during the first week of virus induction. We randomized four weanling female BBDR rats into each of the following treatment groups: (Group A) vehicle alone, (Group B) rrLeptin alone, (Group C) vehicle + pIC + KRV (VPK) and (Group D) rrLeptin + pIC + KRV (LPK). We followed a similar experimental protocol as the one previously described (**Figure 24)**. On day -3, prior to treatment initiation, peripheral blood from rats was collected in heparinized tubes and analyzed by flow cytometry for selected cell populations. Following the bleed, we inserted double alzet pumps (day -3) delivering either vehicle control or 1.2 µg/g/day of rrLeptin into rats in the appropriate groups. Six hours later, four rats in each of VPK and LPK groups were given 1 µg/g body weight of pIC i.p. These rats were treated for two more consecutive days with i.p. pIC followed by a single dose of 10^7 pfu of KRV i.p. (day 0). Alzet pumps were replaced in all treatment groups on days 1 and 6 (post KRV). Rats in all groups were bled for analysis of their PBMC populations on days -1, +3 and +7.

We assessed changes in the frequencies of T cells, T cell subsets, NK cells, NKT cells, B cells and DC/MACs in the peripheral blood on days -3, -1, + 3, and + 7. All cell frequencies are based on live populations only (live/dead blue negative). The frequencies of TCR+ cells decreased by 12% in the VPK group when compared with the vehicle only group on days +3 and +7, but otherwise did not differ significantly between groups (**Figure 42, Table 9**). There was an increase in CD4+ frequencies and decrease in CD8+ frequencies, which increased the CD4:CD8 ratio, in the VPK and LPK treated groups on day -1 and day +7,

when compared with vehicle and rrLeptin only treated rats (**Figure 42, Table 9**). On day -1 LPK and VPK groups had thus far received 2 doses of pIC cells (during the previous two days), but had not yet received KRV. Therefore, we surmise that the increase in the CD4+ to CD8+ ratio on day -1 in the VPK and LPK treated rats may be attributable to the effects of pIC (**Figure 42, Table 9**).

There was an approximately two-fold decrease in the frequency of activated CD4+CD25+ cells, and a two fold increase in the activated CD8+CD25+ populations of the VPK and LPK treated rats compared with vehicle or rrLeptin alone treated rats on day +7. This shift is most likely attributable to the effects of KRV in the VPK and LPK groups than in the vehicle and rrLeptin alone groups (**Figure 43, Table 10**). The increase in frequency of CD8+CD25+ cells in the blood of VPK and LPK treated rats on day +7 corresponded with the observed increase the CD8+CD25+ frequency in the spleens in each of these two groups on day +6 from our previous experiments (**Figure 32**).

We did observe that the frequency of NK cells (defined as all TCR- NKRP1-A+) was significantly lower in the rrLeptin alone, VPK and LPK treated rats compared with vehicle only treated rats on days -1 and +3. Although these trends were the same on day +7, there were no significant differences between groups on this day due to the high variance within groups (**Figure 44, Table 11**). Both rrLeptin and pIC may be responsible for the decrease in the NK frequency since the rrLeptin only group was not treated with pIC, and the VPK group was not treated with rrLeptin, and both these groups experienced a decrease in the NK population relative to vehicle only controls. The frequency of NKT cells was also significantly lower in the VPK and LPK treated rats compared with vehicle or rrLeptin alone

treated rats on days -1, +3 and +7. This effect may be due to the effects of pIC + KRV in these groups. We did not observe any dramatic shifts in the DC/MAC or B cell populations within a group or between groups on any given day (**Figure 45**).

Figure 42. Changes in the Peripheral Blood T Cells Following rrLeptin Treatment

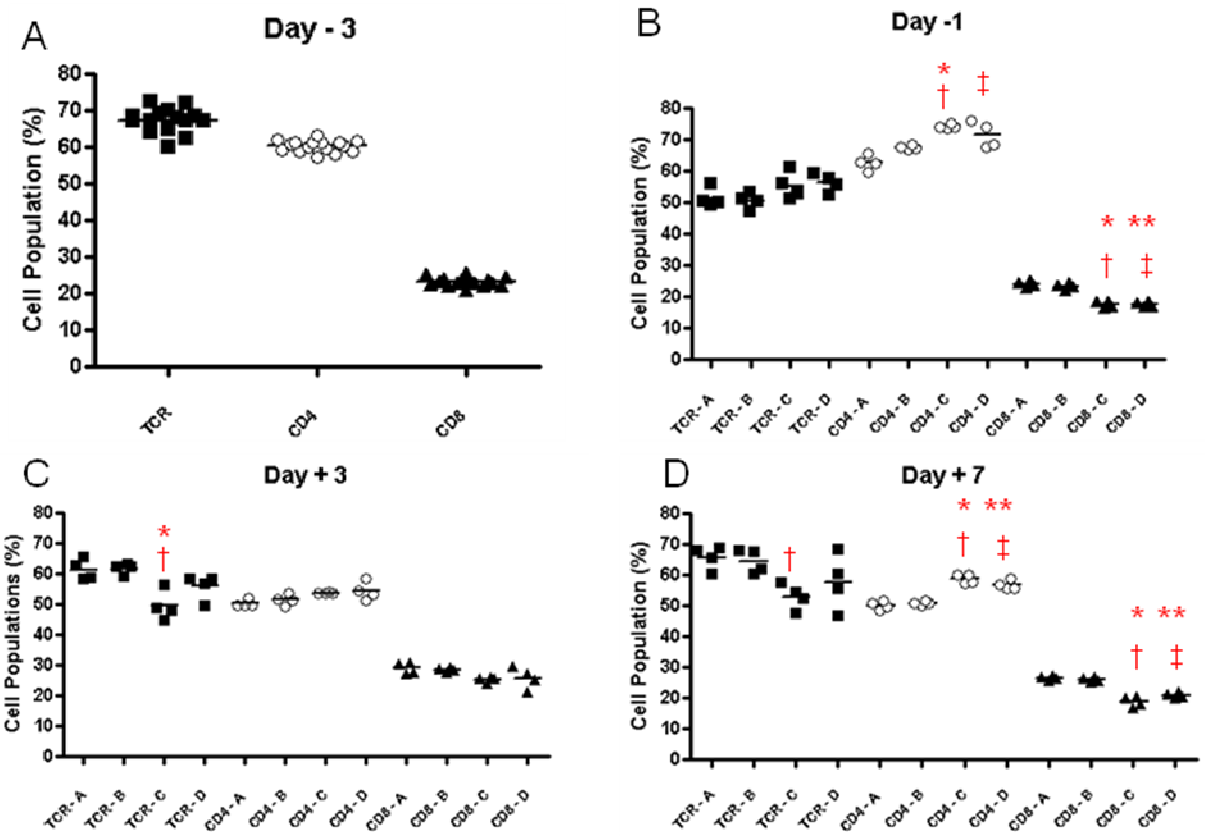


Figure 42. Changes in the frequency of total TCR+, CD4+, CD8+ T cells in peripheral blood of BBDR rats treated with vehicle alone (Group A), rrLeptin alone (Group B), vehicle + pIC + KRV (Group C), and rrLeptin + pIC + KRV (Group D) on day -3 (A), day -1 (B), day + 3 (C) and day + 7 (D) of the treatment protocol. TCR+ cells were gated following selection of the live lymphocyte population (live/dead blue negative). CD4+ and CD8+ cells were then gated following selection of the total live TCR+ population. Statistical analysis included ANOVA followed by Bonferroni comparison of groups (Table 9).

Table 9. Statistical Analysis of Comparisons of T Cell Populations in PBMCs

Comparison Groups	Symbol	Treatment Day	Cell Type	Mean Difference	P value
A vs. C	†	Day -1	CD4	-11.58	P < 0.001
A vs. D	‡	Day -1	CD4	-8.825	P < 0.01
B vs. C	*	Day -1	CD4	-6.8	P < 0.05
A vs. C	†	Day -1	CD8	6.55	P < 0.001
A vs. D	‡	Day -1	CD8	6.55	P < 0.001
B vs. C	*	Day -1	CD8	5.8	P < 0.001
B vs. D	**	Day -1	CD8	5.8	P < 0.001
A vs. C	†	Day +3	TCR	11.8	P < 0.01
B vs. C	*	Day +3	TCR	12.25	P < 0.01
A vs. C	†	Day + 7	TCR	12.73	P < 0.05
A vs. C	†	Day + 7	CD4	-8.5	P < 0.001
A vs. D	‡	Day + 7	CD4	-6.5	P < 0.001
B vs. C	*	Day + 7	CD4	-7.95	P < 0.001
B vs. D	**	Day + 7	CD4	-5.95	P < 0.001
A vs. C	†	Day + 7	CD8	7.775	P < 0.001
A vs. D	‡	Day + 7	CD8	5.6	P < 0.001
B vs. C	*	Day + 7	CD8	7.4	P < 0.001
B vs. D	**	Day + 7	CD8	5.225	P < 0.001

Figure 43. pIC + KRV Induces Increases in Frequency of Peripheral Blood CD8+CD25+ Populations while Decreasing CD4+CD25+ Populations on Day 7

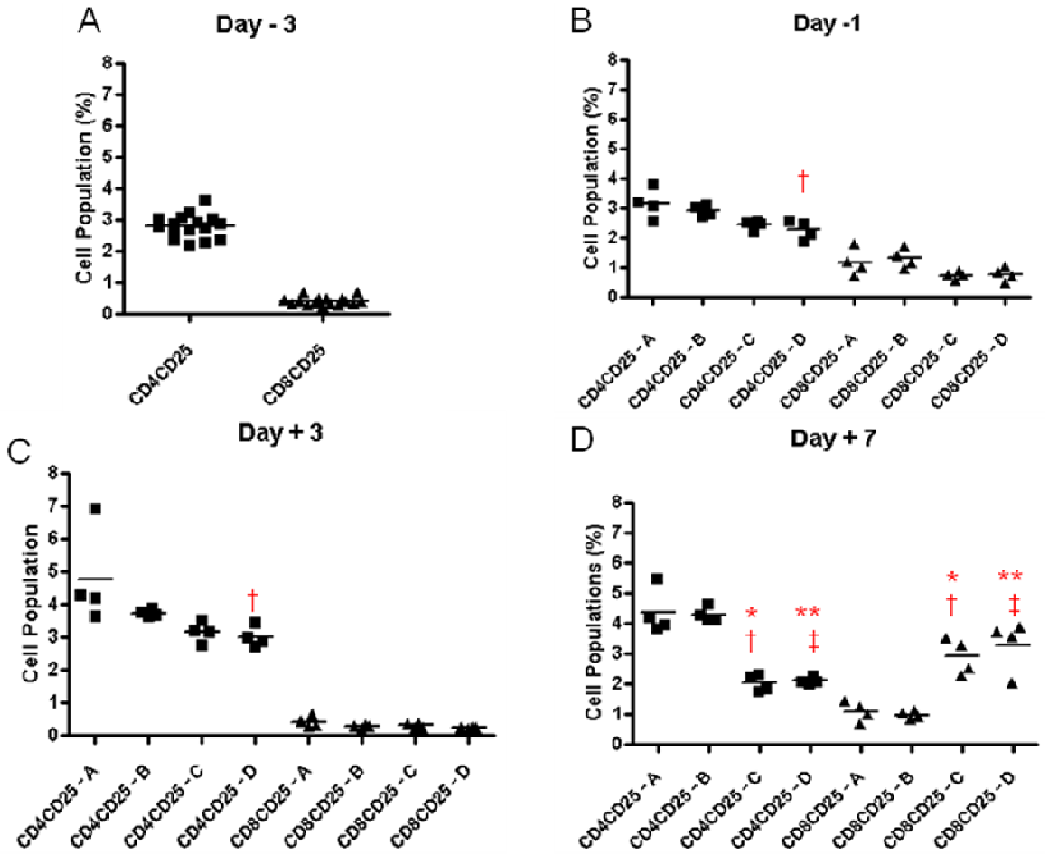


Figure 43. Changes in the frequency of total CD4+CD25+ and CD8+CD25+ T cells in peripheral blood of BBDR rats treated with vehicle alone (Group A), rrLeptin alone (Group B), vehicle + pIC + KRV (Group C), and rrLeptin + pIC + KRV (Group D) on day -3 (A), day -1 (B), day + 3 (C) and day + 7 (D) of the treatment protocol. Subsets of CD4+ and CD8+ cells were gated following selection of the CD4+ or CD8+ cells. Statistical analysis included ANOVA followed by Bonferroni comparison of groups (Table 10).

Table 10. Statistical Analysis of Comparison of CD4+ and CD8+ Subsets in PBMCs

Comparison Groups	Symbol	Treatment Day	Cell Type	Mean Difference	P value
A vs. D	†	Day -1	CD4+CD25+	0.892	P < 0.05
A vs. D	†	Day +3	CD4+CD25+	1.739	P < 0.05
A vs. C	†	Day + 7	CD4+CD25+	2.334	P < 0.001
A vs. D	‡	Day + 7	CD4+CD25+	2.252	P < 0.001
B vs. C	*	Day + 7	CD4+CD25+	2.258	P < 0.001
B vs. D	**	Day + 7	CD4+CD25+	2.175	P < 0.001
A vs. C	†	Day + 7	CD8+CD25+	-1.823	P < 0.01
A vs. D	‡	Day + 7	CD8+CD25+	-2.215	P < 0.001
B vs. C	*	Day + 7	CD8+CD25+	-1.933	P < 0.01
B vs. D	**	Day + 7	CD8+CD25+	-2.325	P < 0.001

Figure 44. Rats Treated with rrLeptin Alone or in Combination with pIC + KRV Decreases Total Peripheral Blood NK Cell Populations

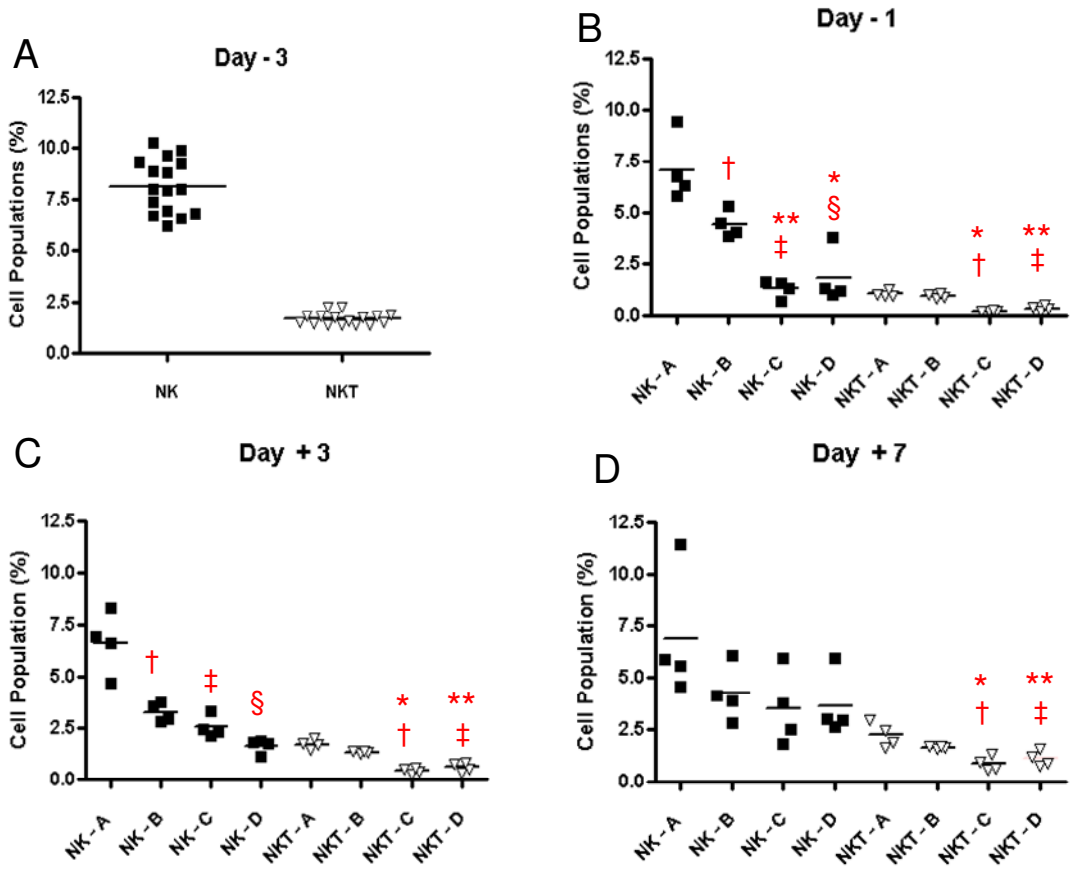


Figure 44. Changes in the frequency of total peripheral blood NK cells (TCR-NKRP-1A+), and NKT cells (TCR+NKRP1-A+) in of BBDR rats treated with vehicle alone (Group A), rrLeptin alone (Group B), vehicle + pIC + KRV (Group C), and rrLeptin + pIC + KRV (Group D) on day -3 (A), day -1 (B), day + 3 (C) and day + 7 (D) of the treatment protocol. Statistical analysis included ANOVA followed by Bonferroni comparison of groups (Table 11).

Table 11. Statistical Analysis of Comparison of NK and NKT Populations in PBMCs

Comparison Groups	Symbol	Treatment Day	Cell Type	Mean Difference	P value
A vs. B	†	Day - 1	NK	2.658	P < 0.05
A vs. C	‡	Day - 1	NK	5.8	P < 0.001
A vs. D	§	Day - 1	NK	5.271	P < 0.001
B vs. C	**	Day - 1	NK	3.142	P < 0.05
B vs. D	*	Day - 1	NK	2.614	P < 0.05
A vs. C	†	Day - 1	NKT	0.8425	P < 0.001
A vs. D	‡	Day - 1	NKT	0.7075	P < 0.001
B vs. C	*	Day - 1	NKT	0.74	P < 0.001
B vs. D	**	Day - 1	NKT	0.605	P < 0.001
A vs. B	†	Day + 3	NK	3.373	P < 0.001
A vs. C	‡	Day + 3	NK	4.102	P < 0.001
A vs. D	§	Day + 3	NK	4.998	P < 0.001
A vs. C	†	Day + 3	NKT	1.275	P < 0.001
A vs. D	‡	Day + 3	NKT	1.105	P < 0.001
B vs. C	*	Day + 3	NKT	0.9	P < 0.001
B vs. D	**	Day + 3	NKT	0.73	P < 0.001
A vs. C	†	Day + 7	NKT	1.39	P < 0.01
A vs. D	‡	Day + 7	NKT	1.14	P < 0.01

Figure 45. rrLeptin Treatment Does Not Cause Dramatic Shifts in the Peripheral Blood DC/MAC and B Cell Populations

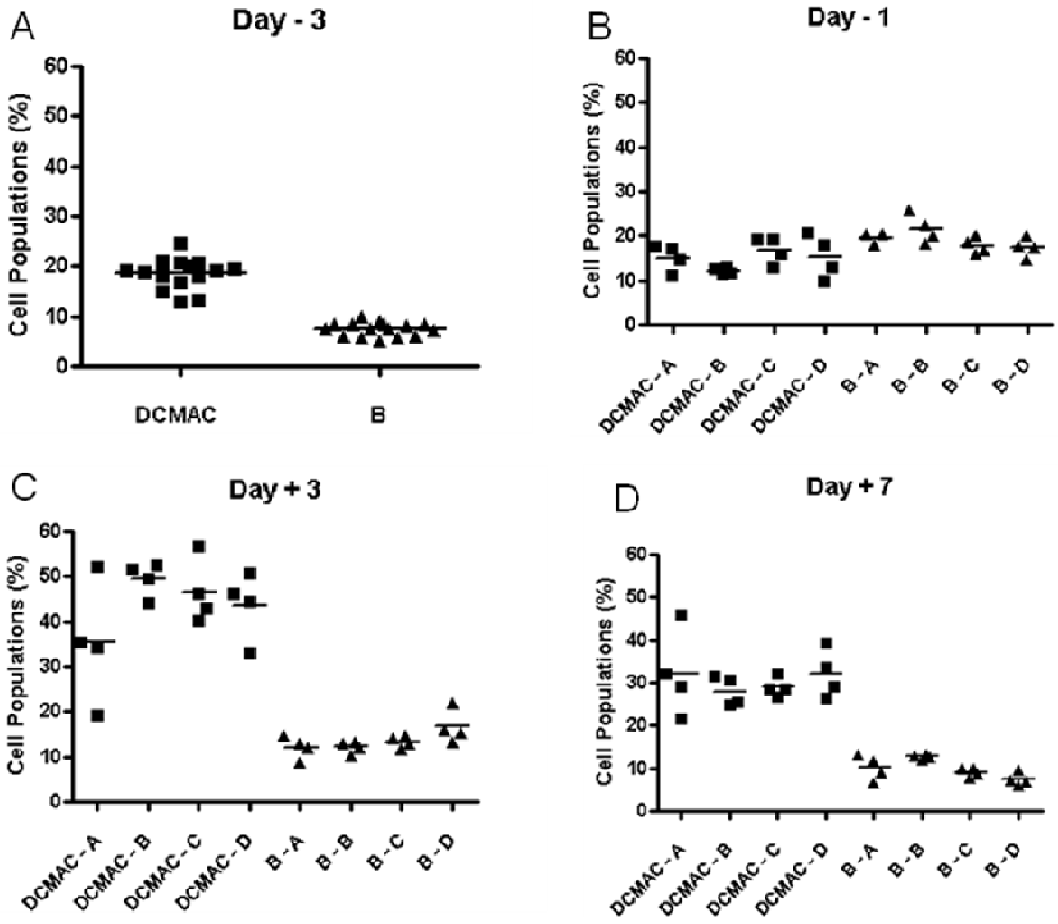


Figure 45. Changes in the frequency of total peripheral blood DC/MACs (CD11b/c+) and B cells (CD45RA+) of BBDR rats treated with vehicle alone (Group A), rrLeptin alone (Group B), vehicle + pIC + KRV (Group C), and rrLeptin + pIC + KRV (Group D) on day -3 (A), day -1 (B), day + 3 (C) and day + 7 (D) of the treatment protocol.

5. *In vitro Stimulation of Splenocytes for 72 Hours with Increasing Concentrations of rrLeptin Results in Decreased Lymphocyte Proliferation at High Concentrations of Both rrLeptin and anti-CD3 and anti-CD28.*

We were concerned that the low body weight associated with hyperleptinemia might have induced a "starvation-like state" that dampened the immune system by preventing the appropriate intake of nutrients necessary for immune cell growth and division. In addition, because the spleen weight to body weight ratio in the LPK treated rats was lower than that of VPK treated rats, we surmised that rrLeptin may have a direct immunosuppressive effect on immune cells of healthy BBDR rats aside from the indirect effects imparted by its anorexigenic properties. To test this hypothesis, we determined the ability of rrLeptin to inhibit T cell proliferation *in vitro.*

Splenocytes from 30-40 day old untreated BBDR rats were labeled with CFSE to identify dividing cells, and stimulated with anti-CD3 and anti-CD28 for 72 hours in culture with increasing doses of rrLeptin (0, 10, 50 and 100 ng/ml). Following 72 hours of culture *in vitro*, there was a dose dependent increase in the proportion of CD4+ and CD8+ cells that proliferated (CFSE lo) in response to anti-CD3 and anti-CD28 in the absence of rrLeptin (**Figures 46a and Figure 46b**). The proportions of CD4+ and CD8+ cells that underwent division when treated with 10 ng/ml of rrLeptin were not different from non-leptin treated cells at any concentration of anti-CD3. The presence of 50 ng/ml or 100 ng/ml of rrLeptin also did not change the proportion of CFSE lo CD4+ or CD8+ cells in splenocytes that were unstimulated or treated with 0.1 µg/ml of anti-CD3. However, at high concentrations of anti-CD3 (1 µg/ml) and anti-CD28 (2.5 µg/ml) we did observe a decrease in the proportion of

CFSE lo CD4+ and CD8+ T cells in splenocytes treated with 50 and 100 ng/ml of rrLeptin. Whether high doses of rrLeptin increased cell death or suppressed proliferation of CD4+ and CD8+ cells at high doses of ant-CD3/anti-CD28 in this *in vitro* culture system will be determined by future experiments.

Figure 46. High doses of rrLeptin Suppress anti-CD3 and anti-CD28 Mediated Proliferation of Splenic CD4+ and CD8+ Cells *In Vitro*

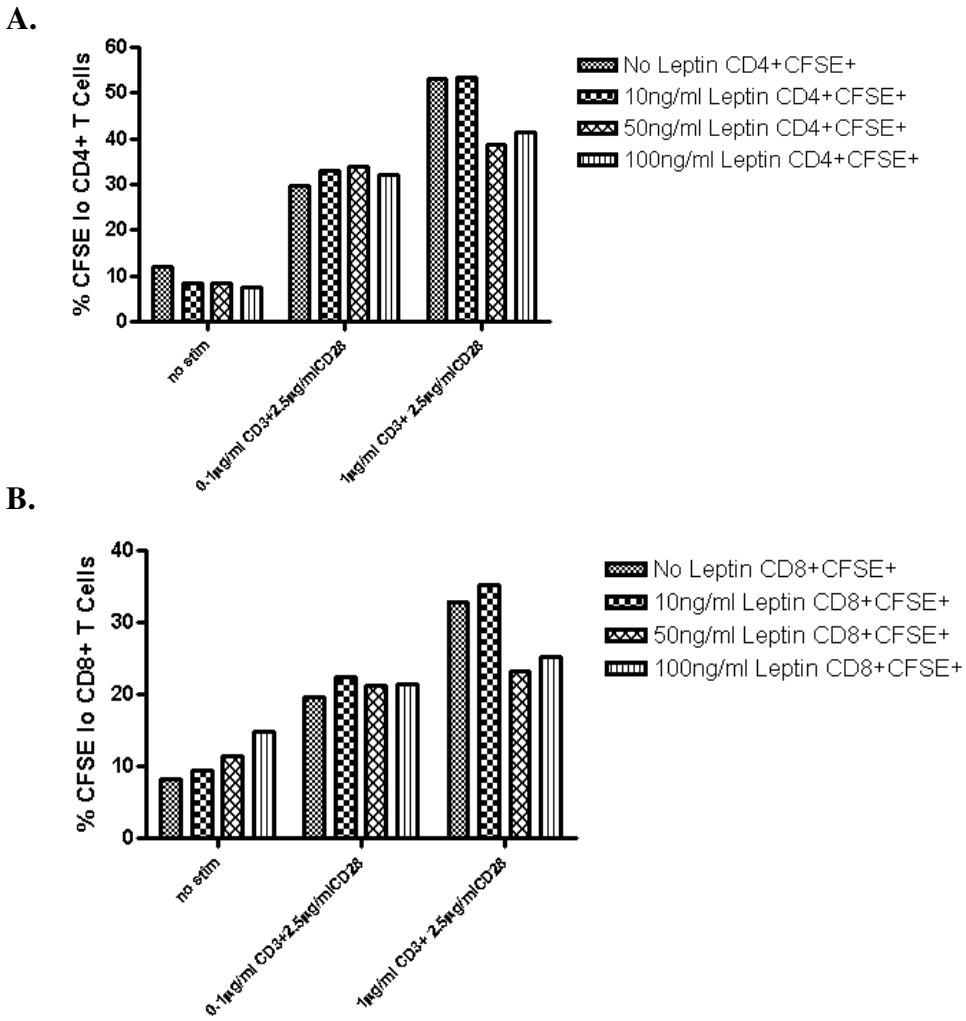


Figure 46. Percentage of splenic (A) TCR+CD4+ T cells and (B) TCR+CD8+ T cells that were CFSE+ compared with CFSE unstained splenocytes at the end of a 72 culture period. Whole splenocytes were cultured with no anti-CD3/anti-CD28 or increasing concentrations of anti-CD3 (anti-CD28 kept constant) in the presence of a range of doses of rrLeptin (none, 10 ng/ml, 50 ng/ml and 100ng/ml).

Summary

An analysis of the splenic populations of female BBDR rats treated with VPK, LPK or vehicle alone has led us to conclude that the hyperleptinemic state generated by rrLeptin promotes a peripheral immunosuppression characterized by a nearly two-fold decline in the number of splenic T cell and APC populations, without radically affecting their proportions. Total spleen weights and the spleen to body weight ratios were significantly lower in LPK than VPK treated rats. While the frequencies of splenic CD4+CD25+ or Treg subsets did not differ between the LPK and VPK groups, both groups treated with pIC + KRV had significantly higher frequencies of CD8+CD25+ T cells than vehicle only controls. However, due to decreased splenic cellularity, LPK treated rats had roughly half as many total CD8+CD25+ cells as VPK treated rats. The increase in frequency of activated CD8+CD25+ cells in the LPK group suggests that hyperleptinemia did not prevent the activation of CD8+ T cells in response to KRV infection. With regard to APC subsets, we did not observe any significant changes in the frequencies of DC/MACs or B cell subsets expressing class II MHC or co-expressing class II MHC and CD86. However, the total number of splenic DC/MACs and B cells expressing class II MHC, and B cells co-expressing class II MHC and the costimulatory molecule, CD86, was lower in the LPK versus VPK treated rats.

Overall, we did not observe any dramatic changes in the frequencies of PBMC cell subsets in LPK versus VPK treated rats. We observed some common effects in the VPK and LPK treated groups, which we attribute to the pIC + KRV treatment. First, there was an increase in the CD4+ to CD8+ ratio on day -1 and +7 in both the VPK and LPK treated rats. Second, there was also an approximately two-fold decrease in the frequency of activated

CD4+CD25+ cells simultaneous with a two fold increase in the activated CD8+CD25+ populations of the VPK and LPK treated rats on day +7. Incidentally, the increase CD8+CD25+ cells in the blood of VPK and LPK treated rats on day +7 corresponded with the observed increase the CD8+CD25+ frequency in the spleens in each of these two groups on day +6. Third, the frequency of NK cells was significantly lower in the VPK and LPK treated rats compared with vehicle or rrLeptin alone treated rats on days -1, +3, and NKTs were significantly lower in these two groups on days -1, +3 and +7. Interestingly, NK cells were also significantly lower in the rrLeptin alone treated group compared with the vehicle only group on days -1, and +3, and trended in that direction on day +7. We conclude from this finding that perhaps hyperleptinemia can independently induce decreases in peripheral blood NK cell populations. No differences in the APC population (DC/MACs or B cells) between any groups were observed in the peripheral blood.

We evaluated the dose-dependent effects of rrLeptin on the proliferative capacity of splenic CD4+ and CD8+ cells from whole splenocytes treated with anti-CD3 and anti-CD28 *in vitro*. We determined that 72 hours following stimulation with 1 µg/ml anti-CD3 and 2.5 µg/ml anti-CD28, high doses of rrLeptin (50 ng/ml and 100ng/ml) were associated with a decreased percentage of CD4+ and CD8+ cells that underwent proliferation (CFSE lo). We conclude from these observations that lymphocytes in whole spleen preparations from normal weight BBDR rats, when exposed to high doses of leptin and strong TCR stimulation, experience an immunosuppressive effect from rrLeptin treatment that is independent of the immunosuppression that may be induced by the anorexigenic, nutrition-depriving properties of rrLeptin.

CHAPTER VI. ROLE OF LEPTIN IN NEW ONSET DIABETES

Introduction

AdLeptin and rrLeptin treatment generated a hyperleptinemic state that could prevent beta cell destruction and autoimmune diabetes. One mechanism by which this cytoprotection may be conferred is via leptin's suppressive effects on the immune system at high serum concentrations. We wondered whether a hyperleptinemic state might also be able to protect residual beta cell mass from an ongoing autoimmune attack in new onset type 1 diabetics. In our virally inducible model of T1D, we observed that at the time of diabetes onset (blood glucose > 250 mg/dl), rats have considerable insulitis in most of their islets. However, some beta cell mass (~10%) is still preserved (unpublished results). If the rats are not treated with insulin pellets upon diabetes onset, they progress to DKA within a matter of three to four days, implying that serum insulin levels become too low to prevent ketosis in the liver. Therefore, there appears to be a lag from the time of onset of disease to when the beta cell mass is completely destroyed. Whether the residual beta cell mass present at the time of diabetes onset is hypofunctional or too small to produce sufficient amounts of insulin to restore euglycemia has not been elucidated in our model. In either case, at the time of diabetes onset in pIC + KRV treated rats, there remains a target beta cell mass that, if protected from the continued onslaught of the immune attack, could perhaps regain its functional capacity to some extent.

Recently, studies using the AdLeptin vector system in new onset NOD diabetics demonstrated that hyperleptinemia could temporarily restore a euglycemic state in these mice

for up to 13 days [102]. Blood glucose levels eventually rose to > 400 mg/dl in AdLeptin treated mice, but never approached the 600 mg/dl range seen in untreated mice. Given this recent evidence regarding leptin's role in reversing a hyperglycemic state in NOD diabetics, we hypothesized that rrLeptin would restore euglycemia in pIC + KRV treated rats with new onset diabetes.

Results

1. *rrLeptin Prevents the Rapid Weight Loss and DKA that Ensues Following Diabetes Onset*

To investigate whether rrLeptin could reverse new onset diabetes, we tested its effects in two experimental studies. In the first study, we treated 21-25 day old male and female BBDR rats with three consecutive doses of 1 µg/g body weight of pIC followed by a single dose of KRV. In the second study, we used three consecutive doses of 2 µg/g body weight of pIC followed by KRV. Seven days following KRV, rats in both cohorts rats were monitored daily for the onset of diabetes. The mean latency to diabetes onset in the first experiment (1 µg/g body weight of pIC) was 15 days, and in the second experiment (2 µg/g body weight of pIC) was 12 days. This difference was statistically significant at 95% confidence interval (Student's T Test, $p = 0.002$). Therefore, we observed that increasing the dose of pIC had an accelerating effect on the time to diabetes onset.

In each of these experiments, we treated rats on the second day of high blood glucose (> 250 mg/dl) with a single 2ML2 alzet pump with either rrLeptin (1.5 µg/g/day) or vehicle control. Rats were monitored daily and sacrificed when they met at least one of these criteria:

(1) loss of ≥ 20% of body weight (2) urine ketone positivity for two consecutive days or (3) moribund appearance. All other rats were followed for 20 days post pump implant, with a single pump replacement on day 10 following the first implant. A schematic diagram of these experiments is depicted in **Figure 47.**

In the 1 µg/g pIC induction protocol, all three vehicle treated rats had significantly shorter survival (based on the criteria mentioned above) than rats treated with rrLeptin (**Figure 48**). The vehicle treated rats lost weight more rapidly than the rrLeptin treated rats (**Figure 49**), and became urine ketone positive within 3.5 days, on average, following the first pump insertion. None of the rrLeptin treated rats became urine ketone positive during the study. Surprisingly, in the absence of exogenous insulin, all three rrLeptin treated rats experienced an immediate fall in their blood glucose levels following pump insertions (**Figure 50**). This decline in blood glucose levels was followed by temporary restoration of euglycemia (BG < 250 mg/dl) in two of three rrLeptin treated rats. One rat experienced restorative euglycemia for up to 18 days, remaining euglycemic at the end of the treatment period. Incidentally, the latency to diabetes onset in this rat was 21 days, which was longer than the average latency for this cohort (15 days), and may have been associated with a larger residual islet cell mass at diabetes onset than the rest of the cohort. The other rat that became euglycemic remained so for 6 days and then reverted to hyperglycemia. The importance of these findings is further emphasized when considering that the reversion to euglycemia in these newly diabetic, rrLeptin treated rats occurred in the absence of exogenous insulin.

Figure 47. Schematic Diagram of rrLeptin Treatment Protocol in New Onset Diabetes

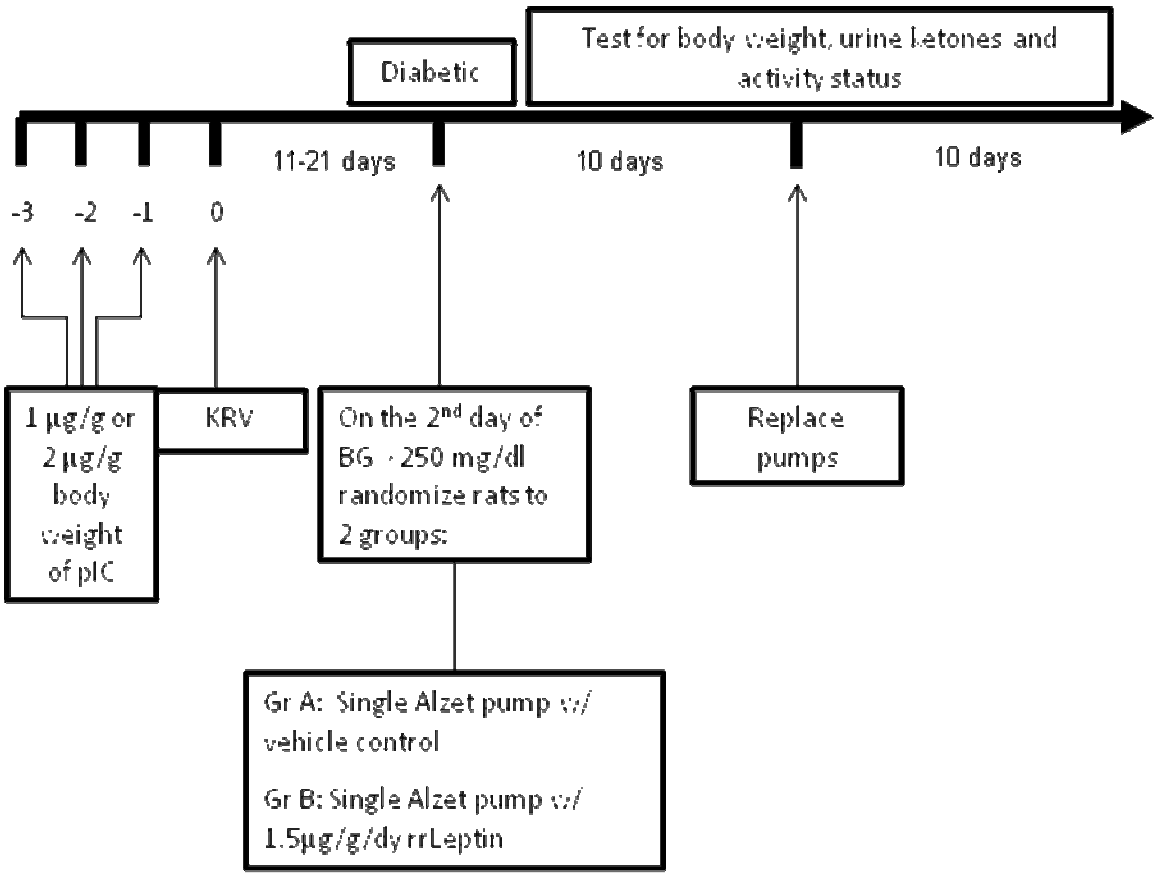


Figure 48. rrLeptin Treated Diabetics Survive in the Absence of Exogenous Insulin

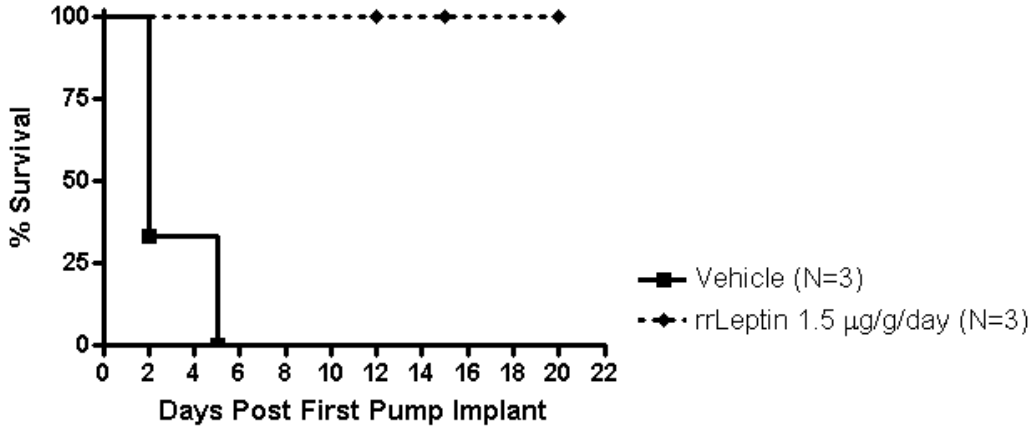


Figure 48. Kaplan Meier curve of the survival of newly diabetic BBDR rats treated with rrLeptin or vehicle control. BBDR rats were rendered diabetic with 1 µg/g body weight of pIC + KRV and treated with alzet pumps delivering vehicle or rrLeptin (1.5 µg/g/day) on the second day of high blood glucose. Rats were monitored for up to 20 days following the first pump insertion. Any rat that met one of three criteria was sacrificed: (1) 20% weight loss (2) urine ketone positivity or (3) moribund status. Pumps were replaced 10 days following the first pump insertion for any rat that survived up to that time point. Statistical comparison of survival curves using the log rank test at the 95% confidence level revealed a significant difference between the groups ($p=0.02$). Median diabetes free survival time in the vehicle control was 3.5 days, while in the rrLeptin group was 20 days.

Figure 49. rrLeptin Causes Less Rapid Weight Loss in New Onset Diabetics than Vehicle Controls

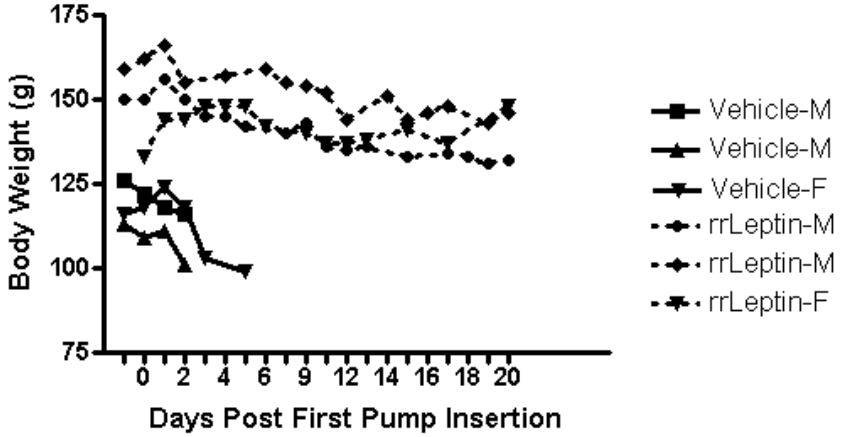


Figure 49. Body weight (g) changes in new onset diabetics (rendered diabetic with 1 µg/g body weight of pIC + KRV) treated with rrLeptin or vehicle control. Data from individual male and female rats in each group is presented due to the differences in the baseline weight of males and females, and also due to the limited sample size in this study.

Figure 50. rrLeptin Temporarily Restores Normoglycemia in New Onset Diabetics

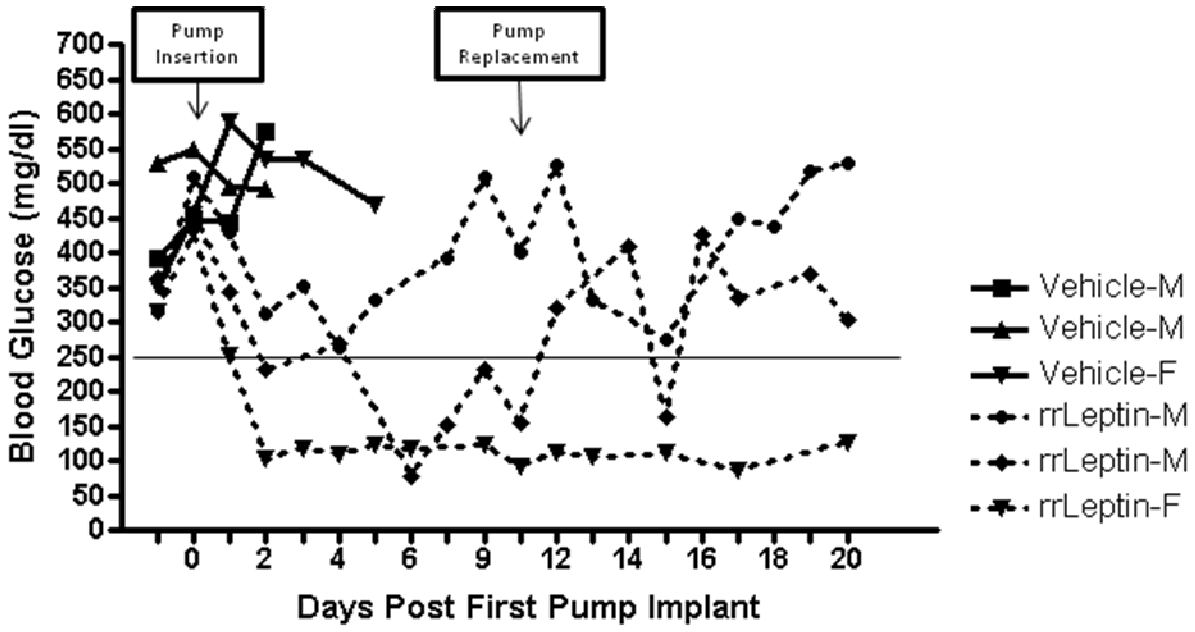


Figure 50. Non-fasting blood glucose changes in new onset diabetics treated with rrLeptin or vehicle control. Data from individual male and female rats in each group is presented due to the limited sample size in this study.

Figure 51. rrLeptin Treated New Onset Diabetics Survive Longer than Vehicle Treated Controls

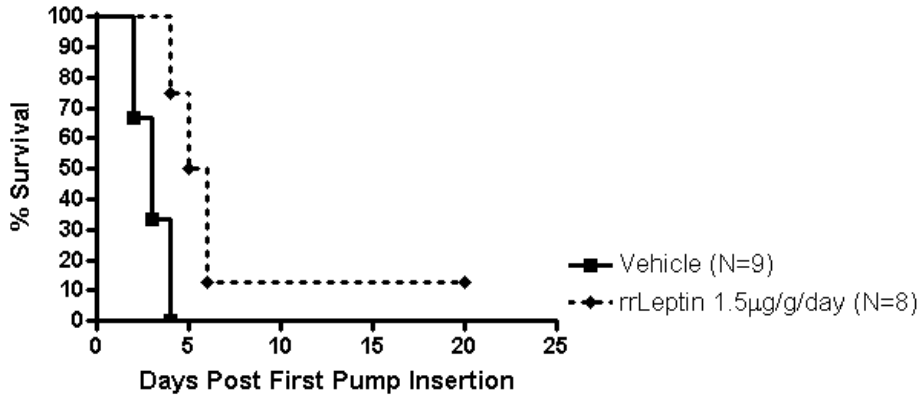


Figure 51. Kaplan Meier curve of the survival of newly diabetic BBDR rats treated with rrLeptin or vehicle control. BBDR rats were rendered diabetic with 2 µg/g body weight of pIC + KRV and treated with alzet pumps delivering vehicle or rrLeptin (1.5 µg/g/day) on the second day of high blood glucose. Rats were monitored for up to 20 days following the first pump insertion. Any rat that met one of three criteria was sacrificed: (1) 20% weight loss (2) urine ketone positivity or (3) moribund status. Pumps were replaced 10 days following the first pump insertion for any rat that survived up to that time point. Statistical comparison of survival curves using the log rank test at the 95% confidence level revealed a significant difference between the groups (p=0.04). Median diabetes free survival time in the vehicle control was 3.5 days, while in the rrLeptin group was 5.5 days.

Figure 52. rrLeptin Causes Less Rapid Weight Loss in New Onset Diabetics than Vehicle Controls

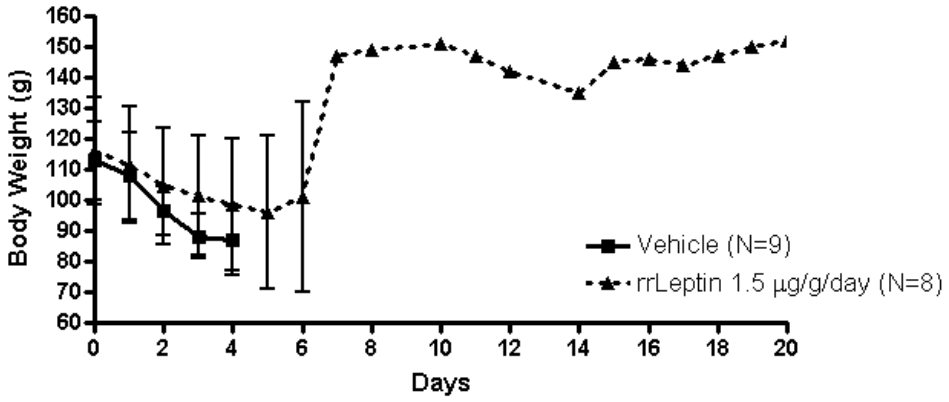


Figure 52. Body weight (g) changes in new onset diabetics treated with rrLeptin or vehicle control. Data from male and female rats in each group is averaged due an equal distribution of males and females in each group. Mean body weights and standard deviations for each time point are represented.

Figure 53. rrLeptin Lowers Hyperglycemia in New Onset Diabetics

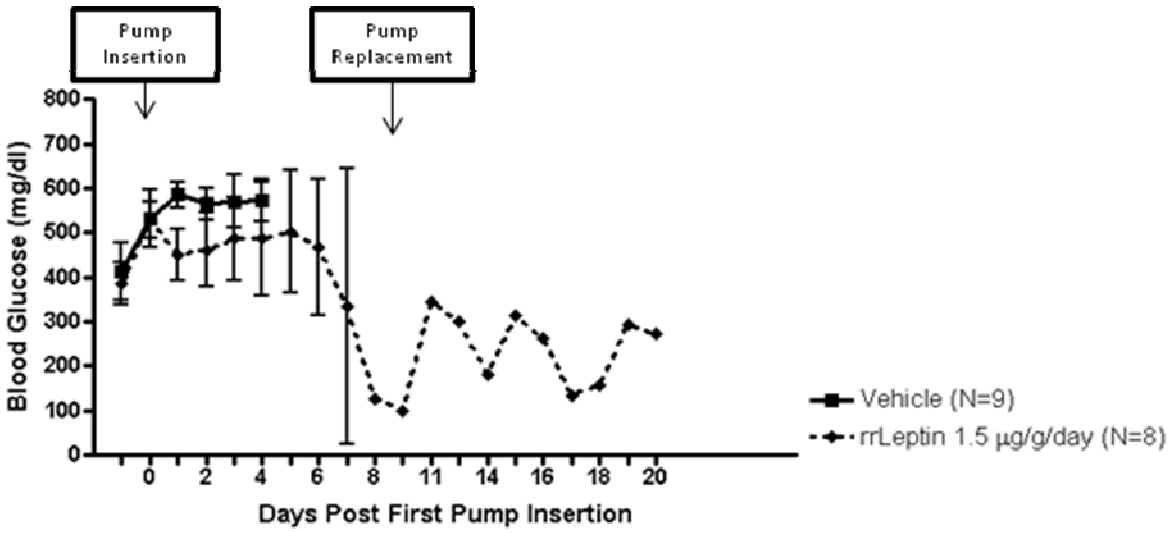


Figure 53. Non-fasting blood glucose changes in new onset diabetics treated with rrLeptin or vehicle control. Data from male and female rats in each group is averaged due an equal distribution of males and females in each group. Mean non-fasting blood glucose levels and standard deviations for each time point is represented.

In our 2 µg/g pIC induction protocol, we observed a similar albeit a less dramatic survival benefit in rrLeptin treated rats compared with vehicle treated controls (**Figure 51**). rrLeptin treated diabetics had a median survival of 5.5 days compared with 3.5 days in the vehicle treated group (p=0.0005, log rank test). One of eight rrLeptin treated rats survived until the end of the treatment 20 day period. The mean latency to onset for this rat was not significantly different from the rest of the cohort. Serum leptin levels in all diabetic rats prior to pump insertion were undetectable, but rose to 37 ng/ml on average in the rrLeptin rats on day 5/6 following pump insertion, while it remained undetectable in the vehicle treated controls.

Vehicle treated controls appeared to lose more weight following pump implantation than rrLeptin treated rats, (**Figure 52**), however, these changes were not significantly different between groups on any day. Average non-fasting blood glucose levels were higher in the vehicle treated controls than the rrLeptin treated rats (**Figure 53**). This result was only statistically significant on the first day following pump insertion ($p<0.0001$, Student's T test). Possibly due to the rapidity of the weight loss in the vehicle treated rats in this experiment (median survival 3.5 days), only four out of nine rats were urine ketone positive at the time of sacrifice. However, similar to the previous study, none of the rrLeptin treated rats became urine ketone positive from the time period following pump insertion until they were sacrificed, despite their hyperglycemic state. We concluded from this observation that perhaps rrLeptin had the capacity to prevent ketosis. However, we would need to assess serum ketone levels in these rats to definitively prove this point. We can say, however, that serum ketones were not high enough in rrLeptin treated rats to appear in the urine.

Furthermore, rrLeptin appears to prevent the rapid weight loss that results from the hyperglycemic osmotic diuresis, which we observed in the vehicle treated controls.

Summary

Our previous studies investigating the role of leptin in virally mediated T1D determined that serum hyperleptinemia could prevent the autoimmune destruction of beta cells that ensues following pIC + KRV treatment. Immune suppression appeared to be at least one modality by which the hyperleptinemic state appeared to protect these rats. We next inquired whether hyperleptinemia could have any effect on the residual islet mass that remains immediately following the onset of diabetes. To answer this question, we rendered male and female BBDR rats diabetic using either 1 or 2 µg/g pIC followed by KRV. On the second day of high blood glucose we randomized rats to receive one alzet pump containing either rrLeptin (1.5 µg/g/day) or vehicle control. We observed that in two of three rats rendered diabetic with 1 µg/g pIC and KRV, rrLeptin was able to restore euglycemia in these rats. Although the euglycemia was only temporary in one of the two rats, in the second rat it lasted until the end of the study (20 days following the first pump insertion). Among rats rendered diabetic with 2 µg/g pIC followed by KRV, which may have a greater beta cell depleting effect at the time of diabetes onset than the 1 µg/g pIC dose, we observed a greater survival benefit among rrLeptin treated rats, and one of the eight rrLeptin treated rats reverted to euglycemia, remaining so until the end of the study. In contrast to the diabetes prevention studies where rrLeptin treated rats failed to gain as much weight as vehicle treated controls, in this study, new onset diabetics treated with rrLeptin failed to lose weight as rapidly as did vehicle treated controls. This apparent dichotomy of action may be explained

by the fact that in normal rats, the anorexigenic properties of leptin prevent weight gain, but in new onset diabetics rrLeptin may helps lower hyperglycemia thereby preventing the rapid weight loss that is commensurate with hyperglycemic osmotic diuresis.

CHAPTER VII. LEPTIN PREVENTS RECURRENCE OF AUTOIMMUNITY IN TRANSPLANTED ISLETS

Introduction

The replacement of insulin production by transplantation of pancreatic islets represents a definitive solution for type 1 diabetics whose endogenous islets have been destroyed by the autoimmune process. Although the establishment of the Edmonton protocol for intrahepatic transplantation of islet grafts has allowed the restoration of euglycemia in diabetics, this restoration is short-lived, requires islets from multiple donors, and necessitates lifelong immunosuppression. With the advent of newer, steroid-free immunosuppressive protocols since 2000, another milestone in islet transplantation has been reached. Now most recipients can become insulin independent without the risks of chronic immunosuppression and remain so for up to a year. Beta-cell function, however, has been discovered to decline thereafter in these patients. Consequently, strategies to enhance the transplanted islet mass and preserve beta-cell function are necessary.

With the successful prevention of autoimmune diabetes as well as the partial reversal of new onset diabetes using rrLeptin in our pIC + KRV model, we asked whether rrLeptin could successfully protect islet grafts transplanted into diabetic BBDR rats from autoimmune recurrence and subsequent graft failure. We hypothesized that a hyperleptinemic state, generated by the continuous infusion of rrLeptin using alzet pumps, would extend the survival of islets transplanted into diabetic BBDR rats.

Results

1. *Effect of Short-Term rrLeptin Treatment on Diabetes Recurrence in Islet Transplanted BBDR Rats*

We initiated a pilot protocol to determine if rrLeptin could prolong islet graft survival in diabetic rats transplanted with syngeneic islets. We first rendered male and female weanling BBDR rats diabetic using pIC (1 µg/g body weight) + KRV. Upon diabetes onset, diabetic rats were injected with a single subcutaneous insulin pellet following the second high blood glucose (> 250 mg/dl). Rats were maintained on the insulin pellet for 7 – 10 days following its insertion to allow for the complete autoimmune destruction of residual islets. Following this time period, insulin pellets were removed on the day of the transplant procedure, and 10 syngeneic islets/g body weight were transplanted under the kidney capsule of each rat. Following islet transplantation, we inserted a 2ML2 alzet pump containing either rrLeptin or vehicle control into the subcutaneous space under the dorsal flank of the skin. Rats were monitored for changes in body weight, blood glucose and onset of ketoacidosis for up to 30 days post transplant. Ten to twelve days following the islet transplantation and initial pump insertion, pumps were replaced in all non-diabetic rats. Thirteen to fifteen days following the pump replacement, pumps were removed entirely, and all non-diabetic rats were monitored for the development of diabetes, defined as two high blood glucose levels (> 250 mg/dl) for two consecutive days. A schematic representation of this protocol is depicted in **Figure 54**.

All three of the rrLeptin treated, islet transplanted rats remained diabetes free until the alzet pumps were removed on day 24 (**Figure 55**). This delay in time to diabetes recurrence

corresponded with a median diabetes free survival time of 30 days in the rrLeptin treated rats. In contrast, three of four vehicle treated rats became diabetic during the follow-up period, which corresponded with a median diabetes free survival time of 17.5 days. At least one vehicle treated rat remained non-diabetic following islet transplantation in our model, suggesting that this animal did not experience a recurrence of the autoimmune destruction of the transplanted graft.

Although rrLeptin appeared to delay the onset to diabetes recurrence in this study, this delay was not statistically significant at the 95% confidence level (p=0.78). Body weight remained constant in both males (N=2), but declined in the female (N=1) rrLeptin treated, islet transplanted rats compared with vehicle control rats, who continued to gain weight following transplantation (**Figure 56**). Non-fasting blood glucose levels in the rrLeptin treated rats also remained low until the pumps were removed on day 24 post transplant (**Figure 57**). The immediate rise in blood glucose levels following removals of pumps in the rrLeptin treated rats suggested that the continuous presence of leptin was necessary to maintain normoglycemia.

Figure 54. Schematic Diagram of the rrLeptin Treatment Protocol in Islet Transplantation Study

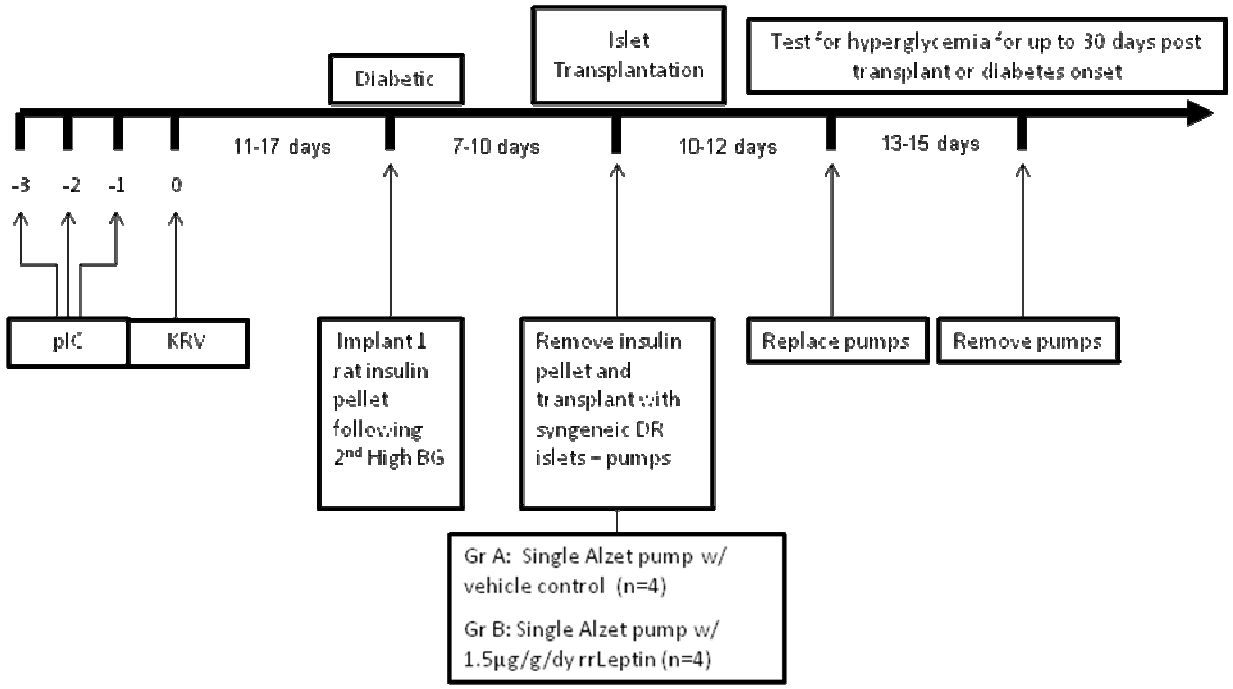


Figure 55. rrLeptin Delays Diabetes Recurrence in Transplanted Islets

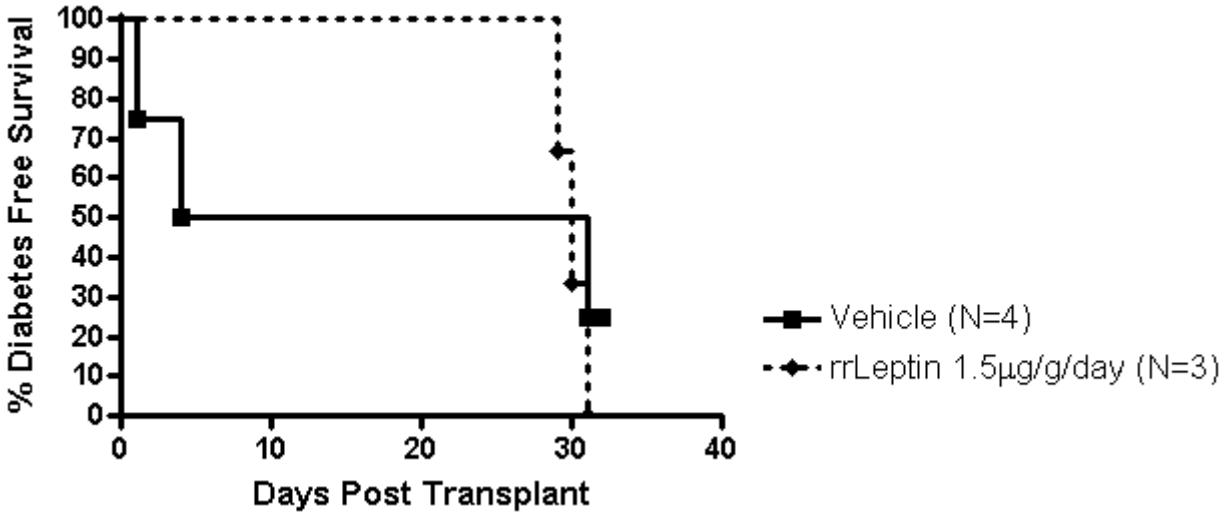


Figure 55. Kaplan Meier curve of the diabetes free survival of transplanted islets into diabetic BBDR rats treated with rrLeptin or vehicle control. BBDR rats rendered diabetic with pIC + KRV were insulin pelleted for 7-10 days prior to islet transplantation and insertion of alzet pumps delivering vehicle or rrLeptin (1.5 µg/g/day) for up to 20 days, with a single pump replacement between days 10-12 and pump removal between days 22-24. Statistical comparison of survival curves using the log rank test at the 95% confidence level revealed no significant differences between the groups. Median diabetes free survival time in the vehicle control was 17.5 days, while in the rrLeptin group was 30 days.

Figure 56. Body Weight Decreases in Islet Transplanted Rats Treated with rrLeptin

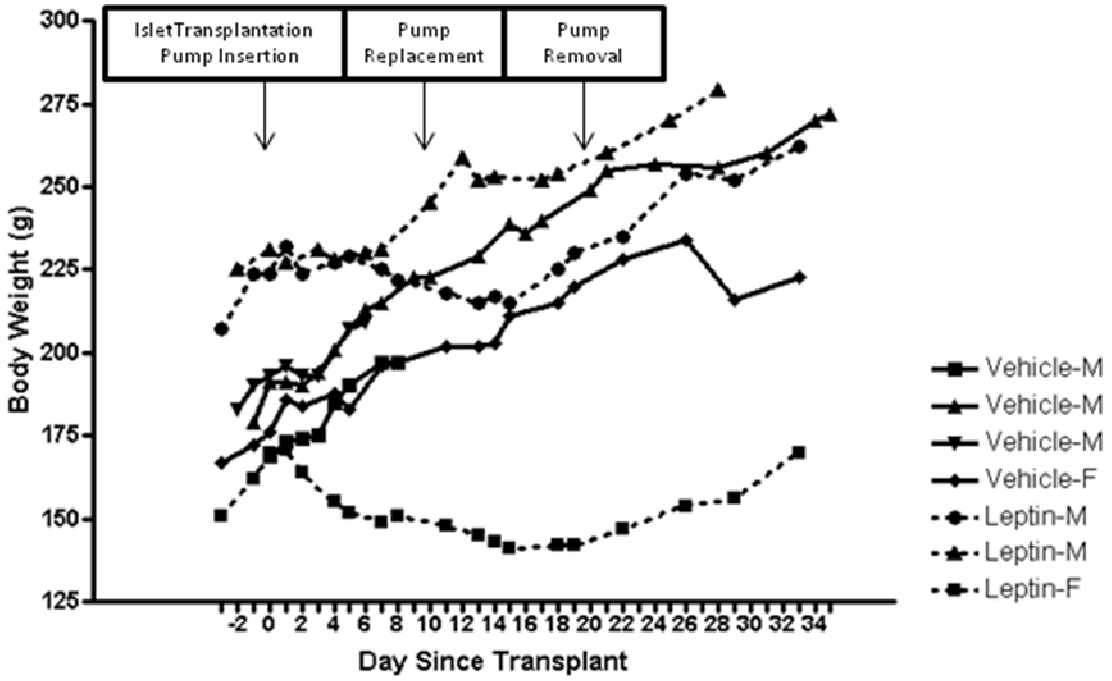


Figure 56. Body weight changes in individual islet transplanted male and female BBDR rats treated with vehicle control or rrLeptin (1.5 µg/g/ day).

Figure 57. Non-fasting Blood Glucose Levels Remain Low in Islet Transplanted Rats Treated with rrLeptin

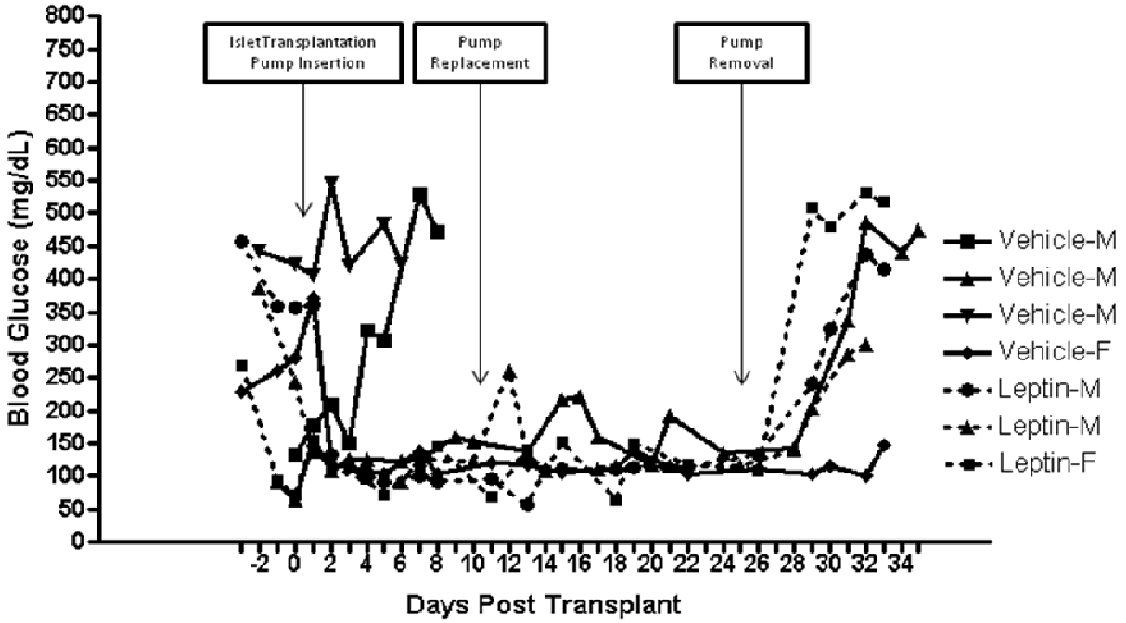


Figure 57. Non-fasting blood glucose levels in individual male and female islet transplanted BBDR rats treated with rrLeptin (1.5 µg/g/day) or vehicle control.

2. Effects of Long-term rrLeptin Treatment on Diabetes Recurrence in Islet Transplanted Rats

To determine if sustaining high serum leptin levels for a longer period of time would result in prolonged islet graft survival, we performed a modified study in which alzet pumps were replaced every 10 days for up to 40 days. Rats were given an insulin pellet for 7 – 10 days after the second high blood glucose (> 250 mg/dl) to allow for complete destruction of residual islets. Insulin pellets were then removed and 10 syngeneic islets/g body weight were transplanted under the kidney capsule of each rat, and a 2ML2 alzet pump containing either rrLeptin or vehicle control was inserted under the dorsal flank of the skin. Pumps were replaced in all non-diabetic rats every ten days thereafter for up to 40 days, for a total of four pump replacements. For up to 50 days post transplant, rats were monitored for changes in body weight, diabetes onset (defined as two high blood glucose levels (> 250 mg/dl) for two consecutive days), and onset of ketoacidosis. At 50 days post transplant, all non-diabetic rats underwent pump removals and uninephrectomy of the kidney containing the islet transplant. Uninephrectomized rats were followed on subsequent days for reversion to hyperglycemia. A schematic representation of this protocol is depicted in **Figure 58**.

We encountered many surgical technical difficulties in performing pump replacements every ten days. The development of infectious seromas or ulcerations at pump sites, resulting from multiple surgical incisions, inevitably forced us to exclude several rats from each treatment group during the course of the study. Of the rats that did not show any sequelae of the multiple surgical manipulations, we obtained data from two rrLeptin treated and six vehicle control treated rats that underwent islet transplantation. Due to the limited sample

size in each cohort, we were unable to observe a statistically significant delay in the time to diabetes recurrence in the rrLeptin treated group (**Figure 59**). However, both rrLeptin treated rats remained diabetes free until 50 days, on which day one of two became diabetic. In the vehicle control treated rats, three of six remained diabetes free until 50 days post transplant. The median survival time in the rrLeptin group and vehicle control groups was 50 days and 27.5 days, respectively. We performed uninephrectomies of the islet transplanted kidneys of both the rrLeptin treated and vehicle treated rats that remained non-diabetic at the end of the 50 day period. These rats became hyperglycemic and remained so for up to three days immediately following the uninephrectomy, indicating that the transplanted and not any remaining endogenous islets were responsible for the glycemic control in non-diabetic rats. The body weights of rats treated with rrLeptin (both males) appeared to be marginally lower (5-10%) than the males in the vehicle control group (**Figure 60**). Blood glucose levels among non-diabetic rats did not differ regardless of the treatment group to which they belonged (**Figure 61**).

The results from the pilot study as well as the present study were highly suggestive that treatment with rrLeptin mediated at least partial protection of transplanted islets. When we pooled the data from both these studies and analyzed the difference in diabetes free survival in the rrLeptin and vehicle treated groups until day 20 post transplant, we observed a difference that approached statistical significance at the 95% confidence level (**Figure 62**, Log rank test, $p = 0.07$). These data suggested that perhaps rrLeptin could protect transplanted islets from autoimmune recurrence. However, since we only observed a 50% recurrence of diabetes in the vehicle control group, we had two potential concerns. First,

rejection of syngeneic, transplanted islets among pIC + KRV induced diabetics may not be a robust model of autoimmune recurrence. Second, surgical manipulations of the rats undergoing multiple pump replacements might have skewed the immune response away from autoimmune recurrence.

Figure 58. Schematic Diagram of rrLeptin Treatment in Islet Transplantation with Multiple Pump Replacements

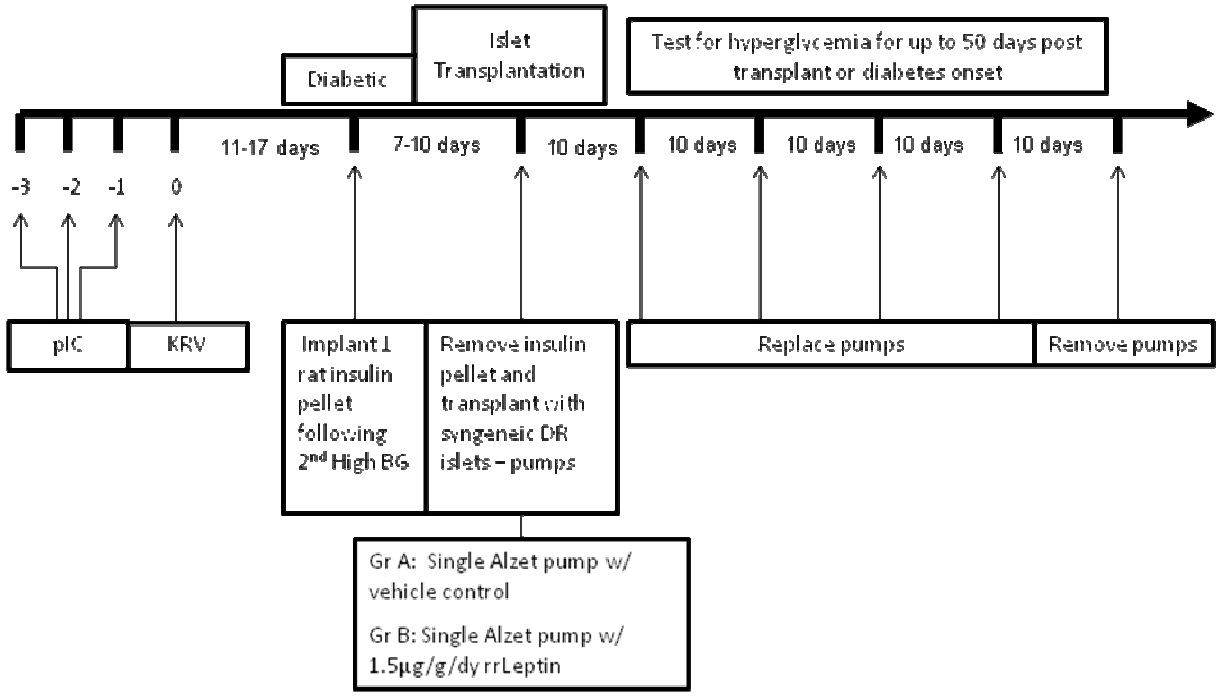


Figure 59. rrLeptin Delays Diabetes Recurrence in Transplanted Islets

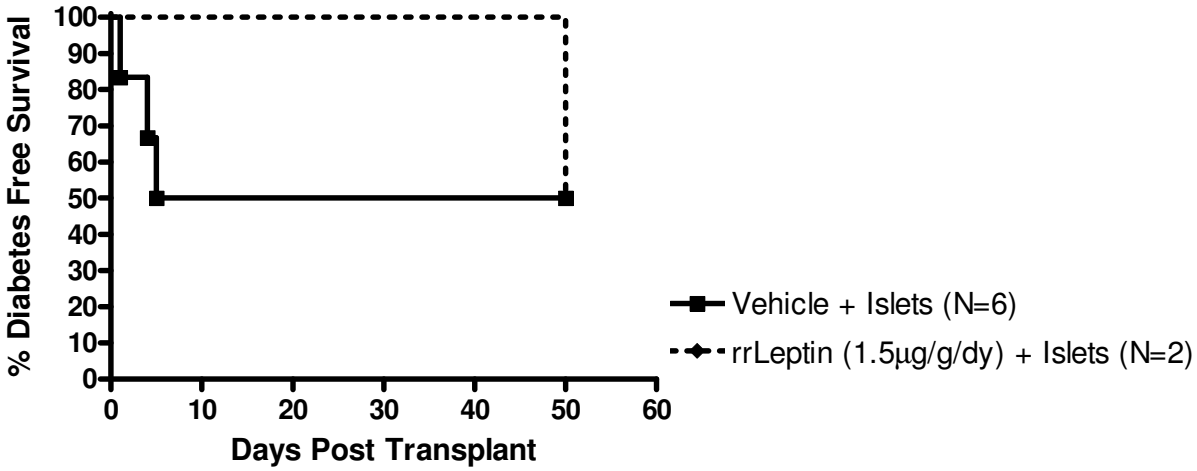


Figure 59. Kaplan Meier curve of the diabetes free survival of transplanted islets into diabetic BBDR rats treated with rrLeptin or vehicle control. BBDR rats rendered diabetic with pIC + KRV were insulin pelleted for 7-10 days prior to islet transplantation and insertion of alzet pumps delivering vehicle or rrLeptin (1.5 µg/g/day) for up to 50 days, with pump replacements every 10 days following the initial pump insertion. Pumps were removed from all non-diabetic rats on day 50. Statistical comparison of survival curves using the log rank test at the 95% confidence level revealed no significant differences between the groups. Median diabetes free survival time in the vehicle control was 27.5 days, while in the rrLeptin group was 50 days.

Figure 60. Body Weight Changes in rrLeptin and Vehicle Control Treated Islet-Transplanted Rats

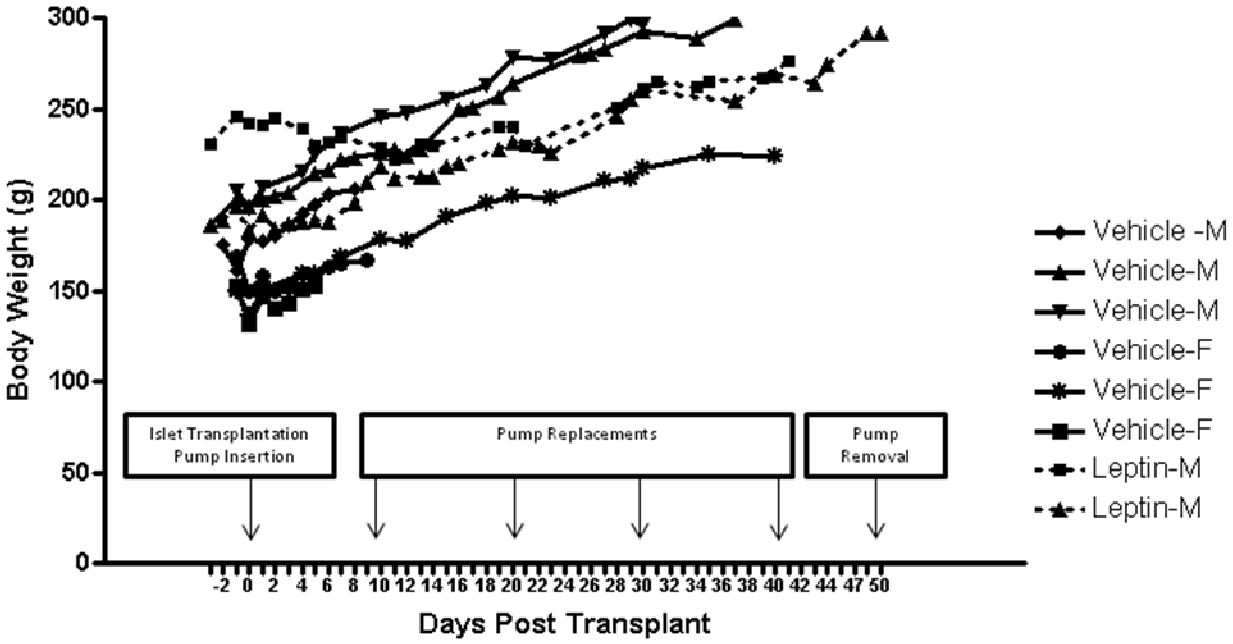


Figure 60. Body Weight Changes in rrLeptin and Vehicle Control Treated Islet-Transplanted Rats.

Figure 61. rrLeptin Treated Rats Maintain Euglycemia Following Islet Transplantation

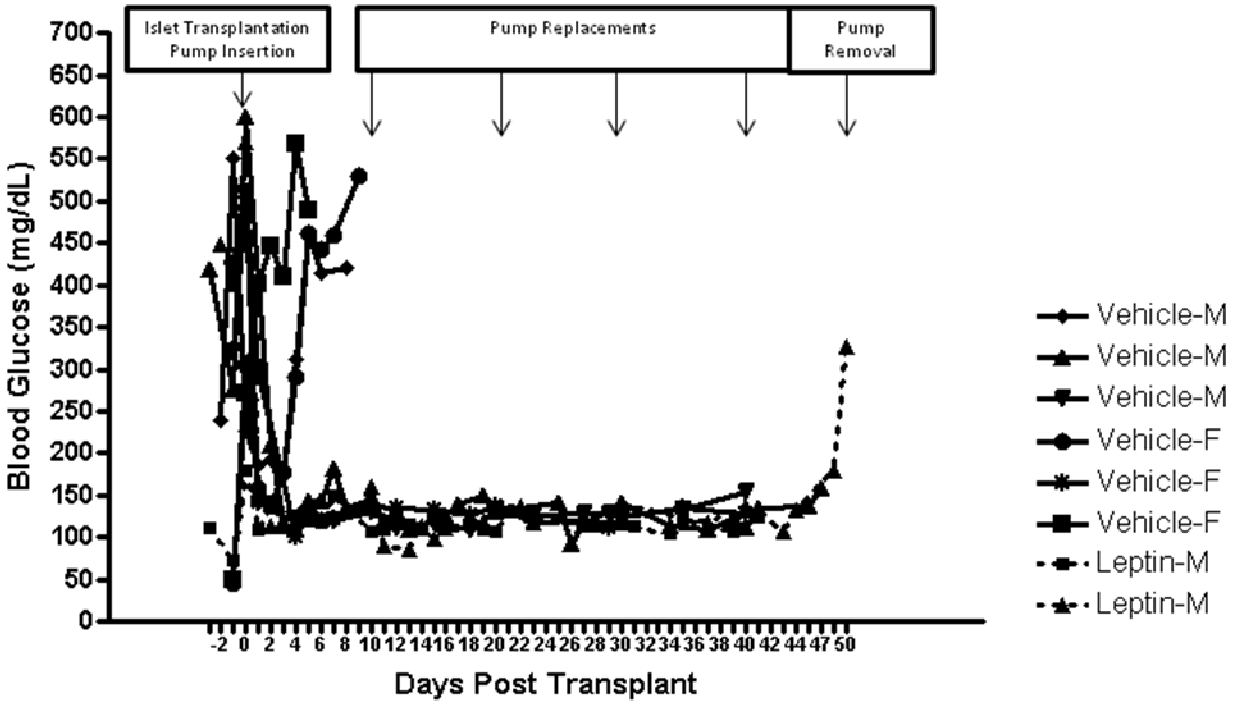


Figure 61. Non-fasting blood glucose levels in individual male and female islet transplanted BBDR rats treated with rrLeptin (1.5 µg/g/day) or vehicle control.

Figure 62. rrLeptin Delays Diabetes Recurrence in Transplanted Islets

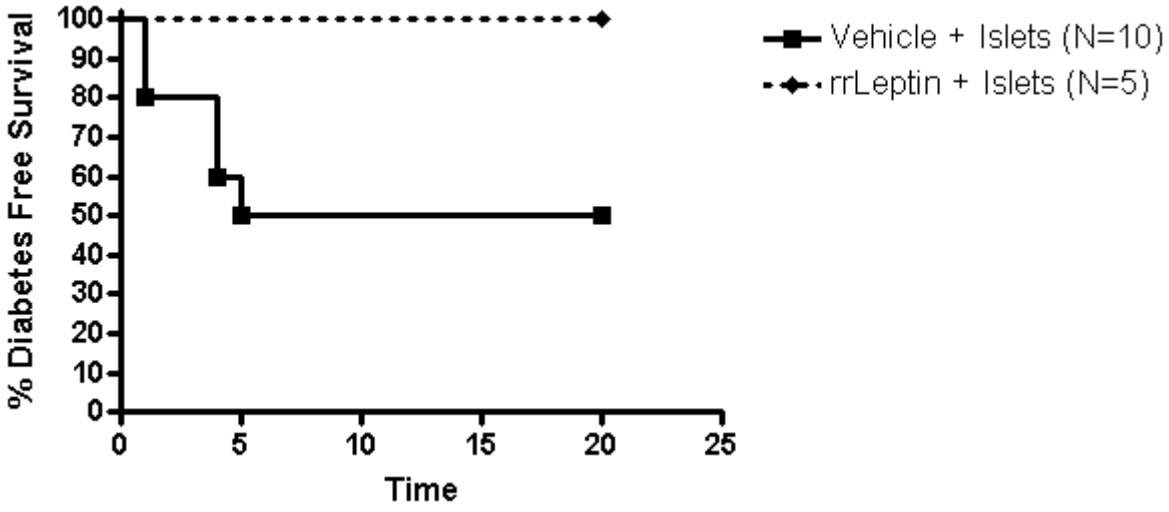


Figure 62. Kaplan Meier curve of the diabetes free survival of transplanted islets into diabetic BBDR rats treated with rrLeptin or vehicle control. Data pooled from two studies using alzet pumps. Both studies replaced pumps on day 10. Statistical comparison of survival curves using the log rank test at the 95% confidence level revealed marginal statistical difference between the groups (p=0.07). Median diabetes free survival time in the vehicle control was 12.5 days, while in the rrLeptin group was 20 days.

3. AdLeptin Protects Transplanted Islet Grafts from Diabetes Recurrence

In previous studies of rrLeptin mediated protection from T1D, we had observed that the use of AdLeptin was as effective as rrLeptin delivered by alzet pumps in protecting rats from T1D. To avoid multiple surgical manipulations with alzet pumps, we employed the use of AdLeptin in the islet transplantation studies. In this protocol (depicted in **Figure 63)**, we rendered male and female BBDR rats diabetic with pIC + KRV. On the second day of high blood glucose (>250 mg/dl) we implanted a single insulin pellet subcutaneously. Three to four days following the insertion of the insulin pellet, rats were treated with 1x 10^{10} pfu per 0.5 ml PBS per 100 g body weight of AdLeptin or AdBetagal i.v. We waited 4-6 days to transplant islets to allow for complete gene expression and generation of a hyperleptinemic state prior to transplantation. On the day of transplantation, insulin pellets were removed and 10 syngeneic islets per gram body weight were transplanted into each rat. Animals in both groups were followed for up to 50 days post transplant for recurrence of diabetes (defined as two consecutive days of blood glucose > 250 mg/dl). Any rat that remained non-diabetic at the end of the follow-up period was uninephrectomized (islet transplant bearing kidney) and monitored for the reversion to hyperglycemia.

Islet transplanted BBDR rats pretreated with AdLeptin experienced significantly longer diabetes free survival than rats pretreated with AdBetagal (**Figure 64**, p=0.01). The cumulative frequency of diabetes recurrence in the AdLeptin group was 10%, compared with 70% in the AdBetagal group, which had a median diabetes free survival time of 26 days. The serum leptin level in a representative subset of rats prior to adenovirus treatment was 0.8 ng/ml ± 0.4 (N=6). Four days after AdLeptin treatment, and immediately prior to islet

transplantation, the serum leptin level in the AdLeptin treated rats rose to 37 ng/ml ± 12.4 (N=6), while among representative AdBetagal pretreated rats, it remained low at 1.4 ng/ml ± 0.5 (N=6). In the one AdLeptin pretreated rat that became diabetic, its serum leptin level prior to islet transplantation was lowest at 16 ng/ml, when compared to the AdLeptin treated rats that remained non-diabetic (mean serum leptin 41 ng/ml, N=5).

Representative body weight data from six rats from each of the AdLeptin and AdBetagal pretreated groups revealed that AdLeptin pretreated male and female rats failed to gain weight during the follow-up period. This lack of weight gain stood in contrast to the AdBetagal pretreated rats all of which continued to experience linear growth, including those that eventually became diabetic (**Figure 65**). Non-fasting blood glucose levels recorded for six representative rats from each of the AdLeptin and AdBetagal group revealed that all non-diabetic AdLeptin pretreated rats were able to maintain normoglycemia, but at times their blood glucose dropped to near hypoglycemic levels (< 50 mg/dl) (**Figure 66**). These occasional episodes of low non-fasting blood glucose levels in AdLeptin pretreated rats seemed coincident with their low body weight and lack of weight gain, suggesting that the lack of nutritional intake may have contributed to the occasional hypoglycemia in this group. All AdLeptin and AdBetagal pretreated rats that remained normoglycemic for up to 50 days reverted to hyperglycemia (blood glucose > 250 mg/dl) for at least two consecutive days following uninephrectomy of the islet-bearing kidney. This reversion indicated that it was the islet graft and not endogenous islets that was contributing to euglycemia.

Figure 63. Schematic Diagram of the AdLeptin Treatment Protocol in Islet Transplantation of Diabetic BBDR Rats

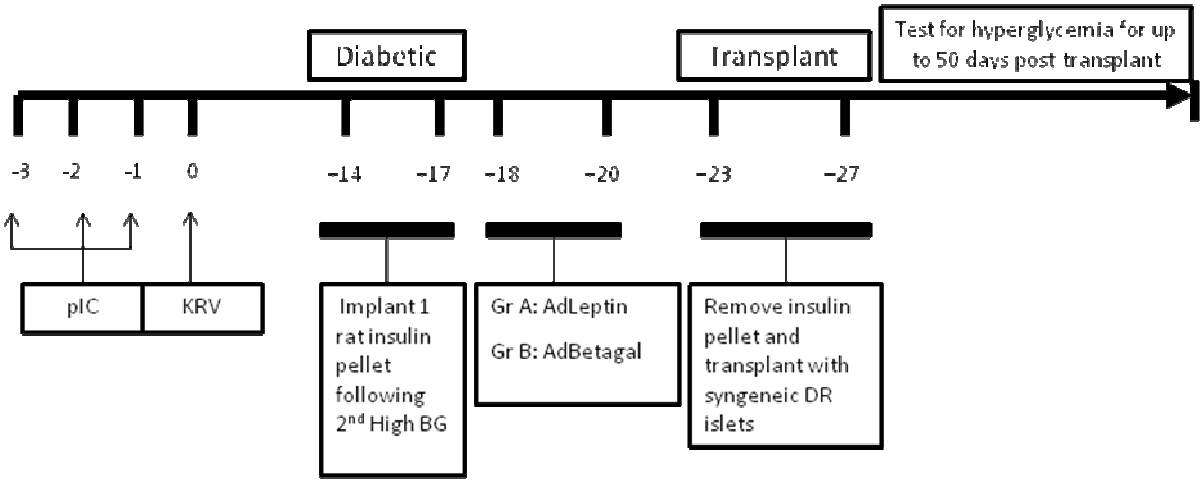


Figure 64. AdLeptin Significantly Protects Islet Grafts from Diabetes Recurrence

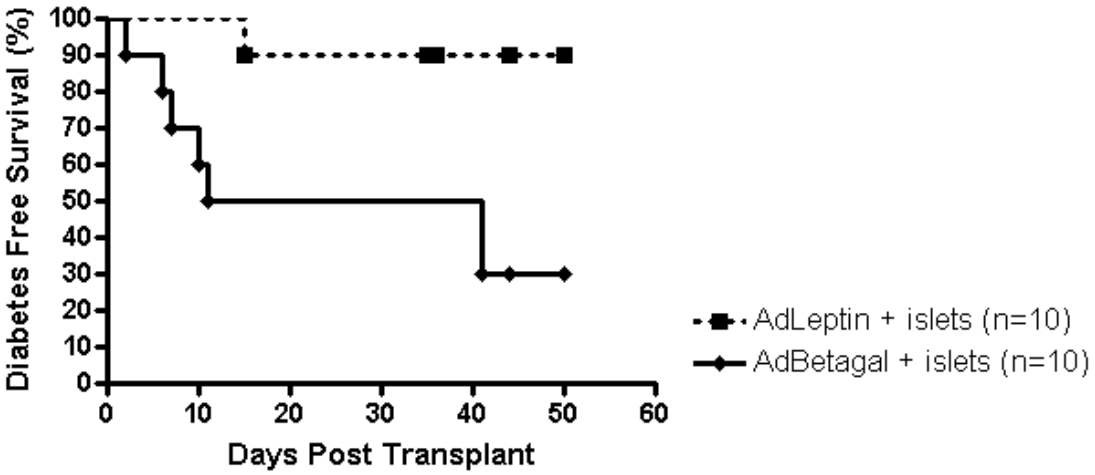


Figure 64. Kaplan Meier curve of the diabetes free survival of transplanted islets into diabetic BBDR rats pretreated with AdLeptin or AdBetagal. Data presented are pooled from two independent experiments. Statistical comparison of survival curves using the log rank test at the 95% confidence level revealed a statistical difference between the groups (p=0.01). Median diabetes free survival time in the AdBetagal group was 26 days, while in the AdLeptin group was 50 days.

Figure 65. AdLeptin Pretreated Rats Fail to Gain Weight Following Islet Transplantation

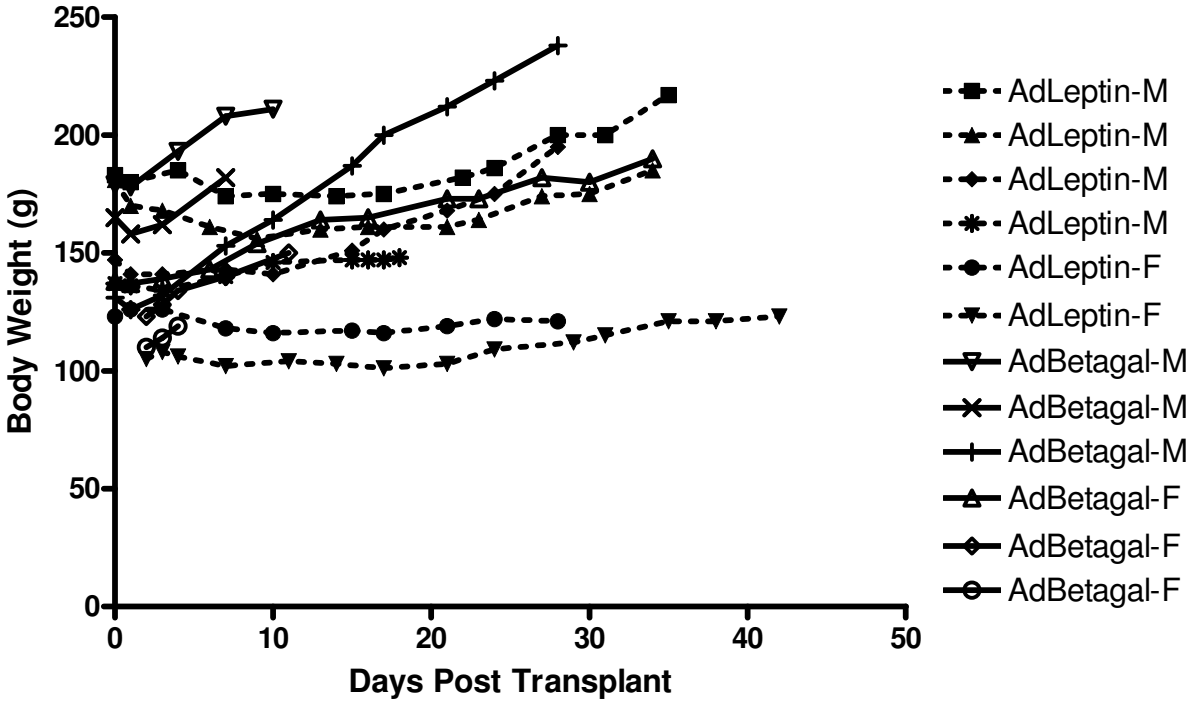


Figure 65. Individual body weight (g) data from six representative male and female rats pretreated with AdLeptin or AdBetagal and given islet transplants under the kidney capsule.

Figure 66. All Non-diabetic AdLeptin Pretreated Islet Transplanted Rats Maintain Normoglycemia During the Study

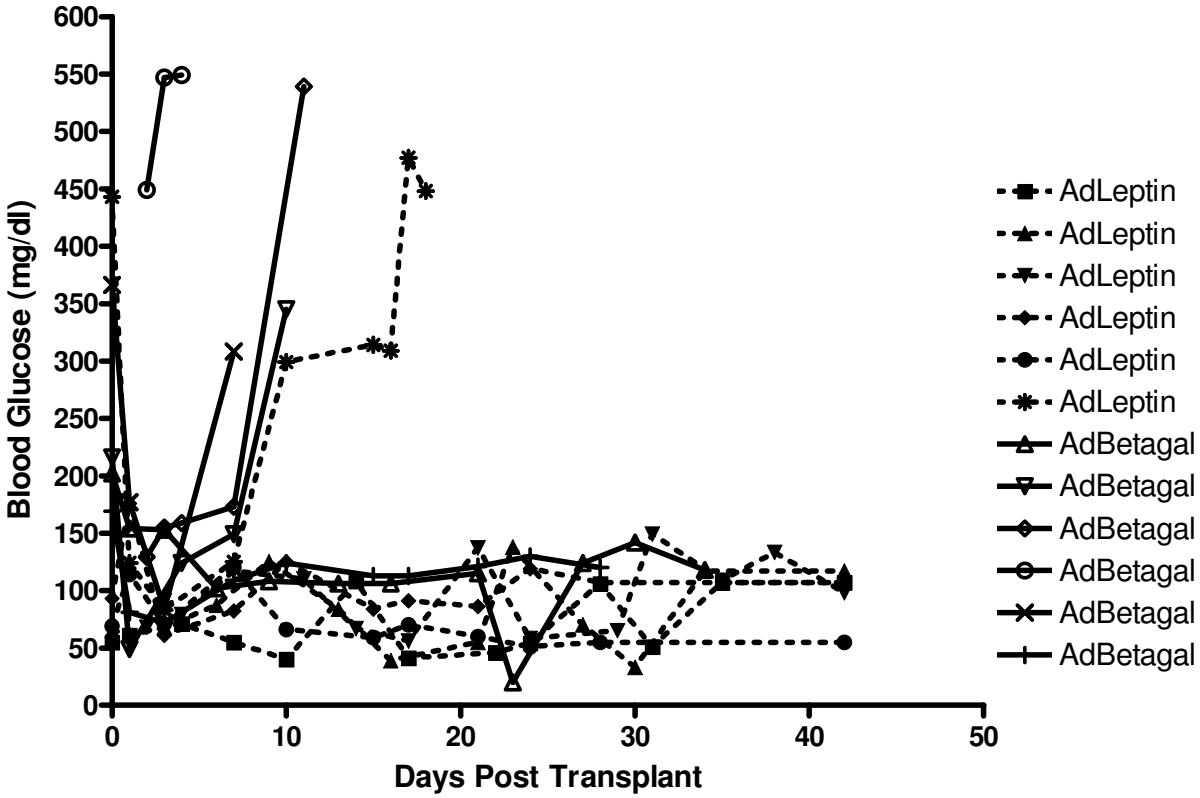


Figure 66. Non-fasting blood glucose levels from six representative AdLeptin and AdBetagal pretreated rats that underwent islet transplantation. Data from individual animals is plotted. Diabetes was defined as two consecutive high blood glucose levels > 250 mg/dl.

Histological analysis of the islet graft in non-diabetic AdLeptin pretreated rats demonstrated that the grafts were intact with positive insulin and glucagon staining (**Figure 67**). The H&E staining did reveal the presence of inflammatory lymphocytes surrounding the islet graft, but their ability to kill beta cells was not apparent due to the abundance of insulin positive beta cells observed in these islets. Pancreata harvested from AdLeptin non-diabetic rats following uninephrectomy of the islet bearing kidney and subsequent reversion to hyperglycemia were also stained for H&E, insulin and glucagon (**Figure 67**). There were no insulin positive cells in the pancreas of these rats, while glucagon staining remained positive. The lack of insulin staining suggested that endogenous islets were not contributing to the euglycemia in these rats during the study.

In contrast, islet grafts from the one AdLeptin pretreated diabetic rat stained positively for glucagon but not insulin (**Figure 68**). As mentioned previously, three of ten AdBetagal pretreated rats remained non-diabetic at the end of the study. Histological analyses of the islet grafts from non-diabetic AdBetagal pretreated rats revealed the presence of infiltrating lymphocytes into the islet grafts by H&E, but positive insulin and glucagon staining (**Figure 69**). In contrast, islet grafts from diabetic AdBetagal pretreated rats revealed some insulin and glucagon staining, but much more sparse than in the non-diabetics (**Figure 70**).

Figure 67. AdLeptin Protected Islet Grafts Are Surrounded by Lymphocytic Infiltrates But Maintain Positive Insulin and Glucagon Staining

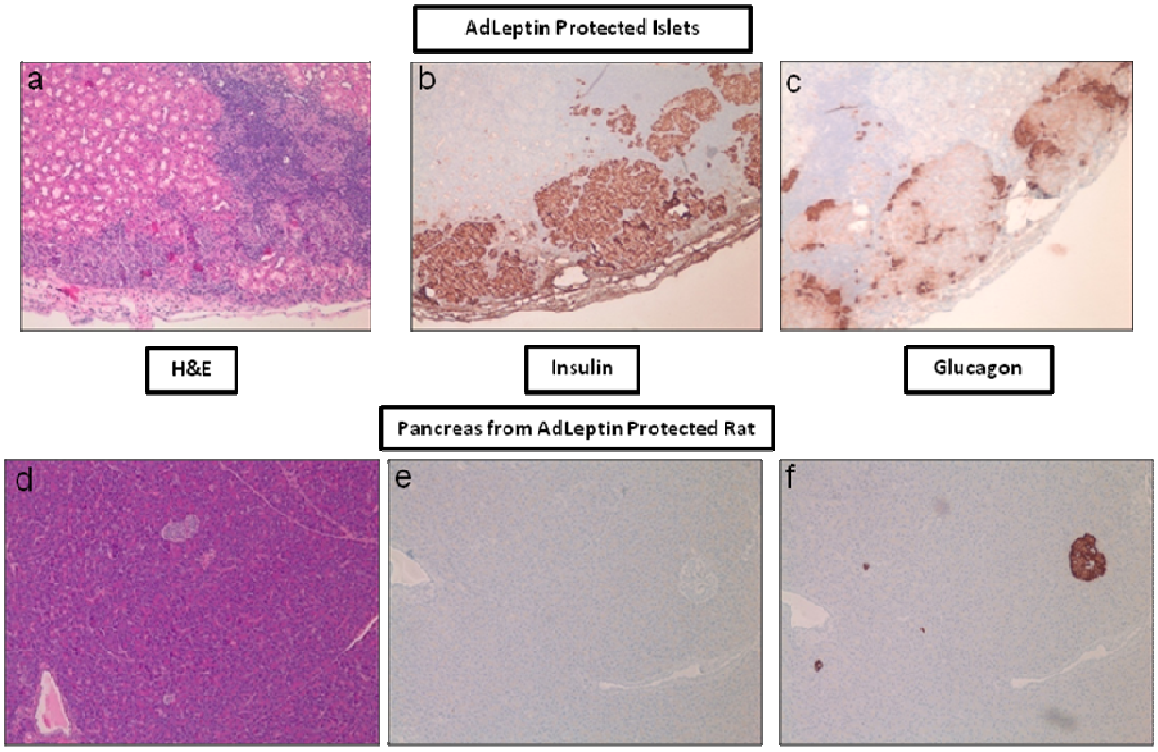


Figure 67. Islet graft (top three panels, 10x magnification) from a representative AdLeptin pretreated, non-diabetic rat was identified under the kidney capsule. Following uninephrectomy and reversion to hyperglycemia of nondiabetic rats, pancreata were harvested for analysis (bottom three panels) Serial sections of each tissue were stained with H&E (top-a, bottom-d), and analyzed by IHC for insulin (top-b, bottom-e) and glucagon (top-c, bottom-f) staining.

Figure 68. Islet Graft from Diabetic AdLeptin Pretreated Rat Does Not Stain Positive for Insulin

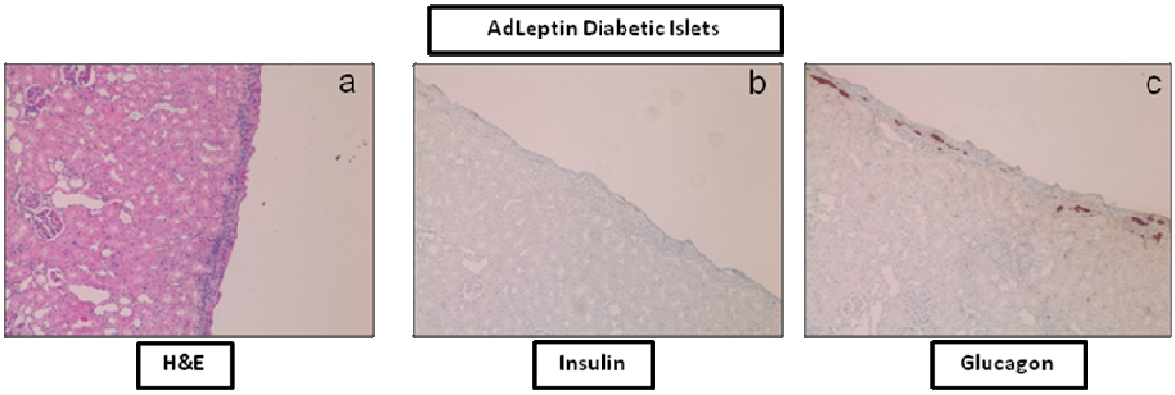


Figure 68. Islet graft from the AdLeptin pretreated, diabetic rat was identified under the kidney capsule. Serial sections of the tissue were stained with H&E (a), and analyzed by IHC for insulin (b) and glucagon (c) staining.

Figure 69. Islet Grafts from Non-diabetic AdBetagal Pretreated Rats Demonstrates the Presence of Insulin Positive Beta Cells

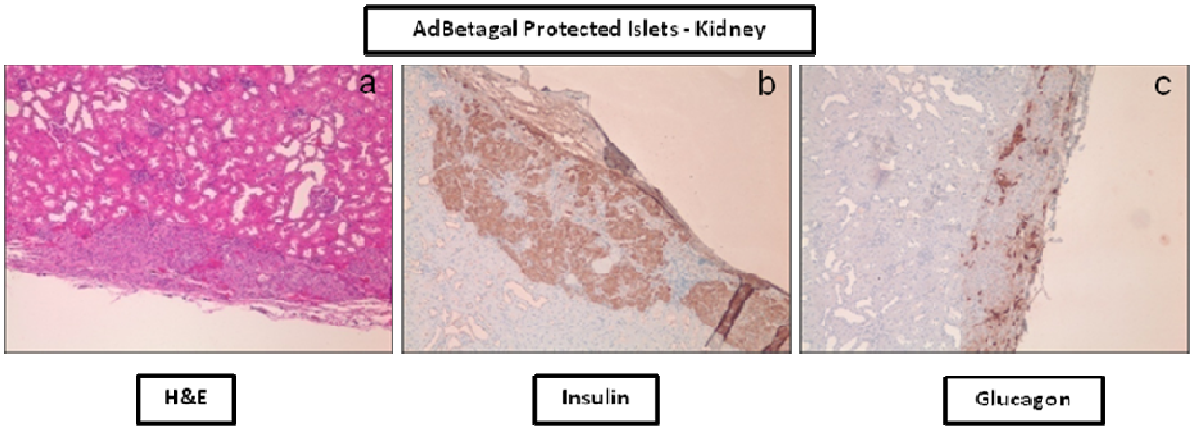


Figure 69. Islet graft from a representative non-diabetic AdBetagal pretreated, diabetic rat was identified under the kidney capsule. Serial sections of the tissue were stained with H&E (a), and analyzed by IHC for insulin (b) and glucagon (c) staining.

Figure 70. Islet Grafts from AdBetagal Treated Diabetics Reveal Minimal Insulin Staining

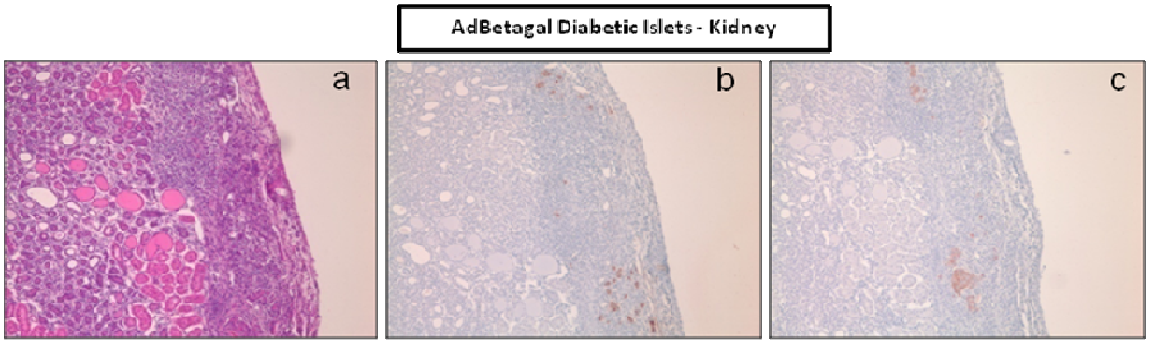


Figure 70. Islet graft from a representative diabetic AdBetagal pretreated, diabetic rat was identified under the kidney capsule. Serial sections of the tissue were stained with H&E (a), and analyzed by IHC for insulin (b) and glucagon (c) staining.

Summary

We conclude that pretreatment with AdLeptin to achieve serum hyperleptinemia prior to islet transplantation was successful in protecting islet grafts from autoimmune recurrence. This method of inducing hyperleptinemia proved to involve fewer surgical manipulations, and their associated immunological changes, than did the use of alzet pumps. We surmise that inflammation and ulcerations from multiple surgical manipulations involved with the alzet pump studies may have altered the survival benefit of rrLeptin. We also observed a slightly larger proportion of AdBetagal pretreated rats transplanted with islets became diabetic (70%) compared with vehicle treated rats in the alzet pump studies (50%). We attribute this difference to the possibility that the adenovirus vector or multiple surgical manipulations modified the immune environment for the recurrence of the autoreactive process. AdLeptin induced hyperleptinemia prevented weight gain, and helped maintain euglycemia in treated rats. Islet grafts from non-diabetic AdLeptin pretreated rats stained positive for insulin and glucagon even though they were surrounded by lymphocytes. This is in contrast to our prevention studies in which minimal or no insulitis was observed in rats pretreated with leptin prior to diabetes induction.

CHAPTER VIII. DISCUSSION

Haptoglobin Is An Early Serum Marker of pIC + KRV Induced Type 1 Diabetes in the BBDR Rat

The need to identify a predictive biomarker that can detect virally induced T1D in humans prompted us to search for such a marker in the serum proteome of a virally induced rodent model of the disease. Causal association between viruses and T1D has been definitively established in rodent models. Nearly 100% of BBDR rats, when deliberately treated with a TLR3 ligand, pIC, followed by a parvovirus, KRV, become diabetic within a reliable time frame (11-21 days). T1D induced by pIC + KRV in BBDR rats shares several similarities with human T1D, which merits its use for global proteome screening techniques. These similarities primarily include lack of a gender bias among diabetics, and risk of similar disease complications, namely DKA. We hypothesized that characterization of the early phases of virus induction in the BBDR rat might reveal differential expression of serum proteins that can serve as fingerprints for the maladaptive, autoreactive process that ensues against the beta cell. To test this hypothesis, sera obtained from rats treated with PBS alone, pIC alone or pIC + KRV on the first day following KRV infection were subjected to two, independent global serum proteomic profiling techniques.

The 2-D gel electrophoresis method identified many acute phase proteins that are involved in heme degradation including haptoglobin, hemopexin, and biliverdin reductase B. Other proteins identified belonged to the serine protease inhibitor superfamily (serpin) including alpha 1 inhibitor III, spin 2a, and alpha 2 macroglobulin (**Table 6**). Alpha and beta

hemoglobin were also identified, pointing to the engagement of the hemolytic pathway in our samples. Of the identified proteins, the most significant differences in protein quantity occurred in the variants of haptoglobin. The isoforms of mature haptoglobin protein (MW ~ 40kDa), were increased up to 9 fold in both pIC alone and pIC + KRV treated rats relative to rats treated with PBS alone**.** With a similar increase in the mature isoform of the protein in both pIC alone and pIC + KRV treated rats compared with PBS, we expected to observe an equivalent rise in the serum levels of the zymogen cleavage fragments in both these groups. However, by image analysis of 2-D gels, we observed that peptide fragments of the prohaptoglobin precursor protein and preprohaptoglobin (MW ~ 13kDa and ~30kDa, respectively) were decreased between 2-3 fold in rats treated with pIC + KRV compared with pIC alone. We attributed these unexpected findings to the following possibilities: (1) artifact of gel processing or (2) peptide suppression during the MALDI-TOF-MS/MS identification process preventing their appropriate identification in the pIC + KRV sample or (3) a biological attribute specific to the pIC + KRV model where precursor fragments are differentially cleared from the serum, and thereby decreased in concentration in the serum.

We applied iTRAQ technology on the same samples used for 2-D gel analysis to independently verify if similar protein patterns would emerge using a complementary global proteome search technique. iTRAQ analysis was able to identify a larger number of differentially expressed proteins than 2-D gel analysis (**Table 7**). Many of the proteins identified were acute phase reactants, serine protease inhibitors, proteins involved in heme degradation, and by-products of hemolysis, as we had observed in the 2-D gel analysis. Of importance to us was the iTRAQ identification of increased levels of haptoglobin precursor

in the sera of pIC alone and pIC + KRV treated rats compared with PBS treated rats. By iTRAQ analysis, it appeared that haptoglobin precursor was 2.7 fold higher in pIC + KRV treated rats than in rats treated with pIC alone.

Despite some incongruencies in the isoforms of haptoglobin identified by 2-D gel analysis and iTRAQ, the further evaluation of haptoglobin as a biomarker was of particular interest to us because of its biological properties. Haptoglobin, an acute phase reactant, has been reported to inhibit hemoglobin-induced oxidative damage in cells, and possesses anti-inflammatory and immunomodulatory properties. It has been demonstrated to inhibit prostaglandin synthesis [103], dampen the inflammatory capacity of neutrophils, monocytes and macrophages [104] and interfere with B- and T-cell mitogenic responses *in vitro* [105]. Additionally, in humans, mutations in the haptoglobin gene are associated with increased risk of diabetic complications such as retinopathy, nephropathy and cardiovascular disease [106, 107]. Therefore, we were interested to determine if elevations in haptoglobin in our diabetes induction model might be a reliable marker for the autoreactive inflammatory response.

We performed several validation studies to determine if haptoglobin levels were sensitive in detecting T1D development. We first tested whether haptoglobin levels were significantly different in pIC + KRV rats, the majority of which become diabetic, compared with rats rats treated with pIC alone or pIC + H-1, neither of which develop diabetes. By both western blot analysis (**Figure 2a**) and ELISA (**Figure 3a**) we determined that haptoglobin levels rose following three consecutive days of treatment with pIC, but remained elevated in the pIC + KRV group while declining in other groups. There were significantly higher serum haptoglobin levels in the sera of pIC + KRV treated rats on day +3 and day +6 following

KRV infection than in rats treated with pIC alone, pIC + H-1 or PBS alone. This result was replicated in our second validation experiment where rats were cardiac bled to reduce the probability of hemolysis in the samples. Again, by both western blot (**Figure 2b**) and ELISA (**Figure 3b**) we observed significantly elevated serum haptoglobin levels on day +3 and day +7 in the rats treated with pIC + KRV compared with other groups. These results implied that perhaps haptoglobin was a sensitive marker for pIC + KRV induced T1D.

We then tested the sensitivity of the biomarker in virus models where T1D expression was variable. KRV alone (without pretreatment with a TLR ligand) causes diabetes in 20-40% of infected BBDR rats. RCMV alone, similarly, causes diabetes in 20-40% of LEW1.WR1 rats. If haptoglobin is a sensitive biomarker for virally induced T1D, its levels should only be significantly elevated in those rats that became diabetic. Unfortunately, due to the small sample sizes in our experiments, we did not see a statistically significant increase in haptoglobin levels among diabetics compared with non-diabetics in either the KRV alone or RCMV alone models. Haptoglobin levels did peak earlier in KRV treated rats that became diabetic than those that did not, but the serum levels were not significantly different between diabetics and non-diabetics on any given day (**Figure 5a**). In the RCMV model, although elevations in serum haptoglobin levels in rats that became diabetic were not significantly different from rats that remained non-diabetic, they trended higher in diabetics than non-diabetics at all time points (**Figure 5b**).

In this report, we provide evidence of haptoglobin as a sensitive biomarker for pIC + KRV induced T1D, and its sustained serum elevations in this model may reveal insight into the pathogenesis of the disease. KRV is known to be strong inducer of pro-inflammatory

cytokines such as IFN-γ and IL-12, and it has been reported to alter lymphocyte responses and cytotoxic lymphocyte activity *in vitro* [52]. A closely related virus, H-1, despite sharing 80-90% sequence homology, does not induce pro-inflammatory cytokines to the same degree [50]. Five days following infection, serum IL-20p40 levels, for example, are highest in KRV treated rats when compared with rats treated with pIC alone or H-1 virus alone. This reported timing of cytokine upregulation appears to correspond with the period during which we observed significant increases in the serum haptoglobin levels in the pIC + KRV treated rats.

KRV and H-1 have been reported to be especially pathogenic to the developing liver and cerebellum [20]. KRV antigens have been known to infect hepatocytes, in addition to T cells and B cells. Haptoglobin is an acute phase response protein secreted by the liver in response to proinflammatory cytokines [108], and perhaps, in the KRV model, in response to direct infection with KRV, although this remains to be proven. Sustained elevations in serum haptoglobin in the pIC + KRV model, may be attempts by the body, albeit ineffective, to guard against excessive inflammation and cytotoxicity generated by this perturbation. In our study we have discovered that treatments, including pIC alone, pIC + H-1, KRV alone, RCMV alone and pIC + KRV, elicited an immediate rise in serum haptoglobin levels relative to PBS treatment. However, sustained elevations in serum haptoglobin, were only observed with pIC + KRV treatment (**Figure 3a, 3b**). These haptoglobin molecules in the BBDR rat model were functional as demonstrated by their ability to bind to hemoglobin moieties and form hemoglobin:haptoglobin complexes (**Figure 4**). Elevated haptoglobin levels, in response to KRV infection, may reveal the chronic inflammatory response of the liver to an environmental perturbant that can eventually precipitate autoimmunity in a susceptible host.

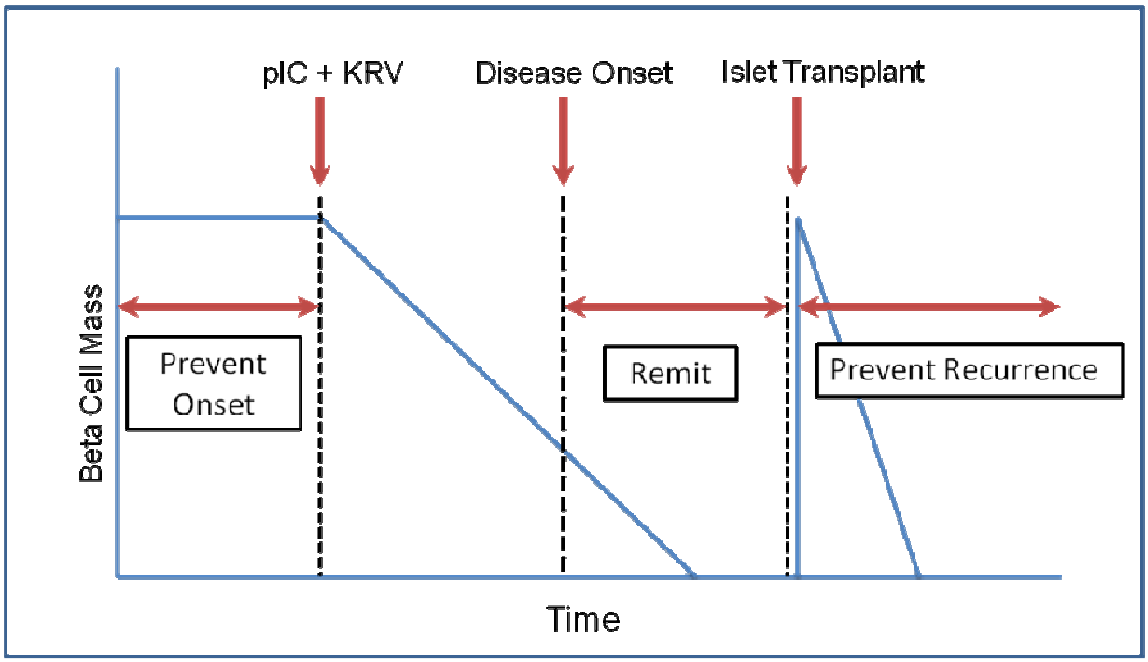
We propose that tracking sustained elevations in serum haptoglobin levels following viral infection, in conjunction with currently used biomarkers, namely autoantibodies, may provide a more robust predictor of virally induced T1D in genetically susceptible children.

Emerging Therapeutic Strategies for the Treatment of Type 1 Diabetes

Insulin therapy is necessary to prevent life-threatening DKA in newly diagnosed type 1 diabetics, but does not cure the disease. The primary goal of emerging therapeutic interventions for T1D, therefore, is the preservation of insulin-secreting beta cells. There are at least three time points at which therapeutic intervention may be feasible **(Figure 71)**. Therapeutic agents can be applied to (1) prevent disease prior to clinical onset, (2) remit disease once it is clinically apparent, and (3) prevent recurrence of disease in transplanted islets. The leading agents being tested in clinical trials for T1D at these defined time points are primarily antigen-specific or antigen-nonspecific immunomodulatory agents aimed at abrogating autoreactivity to the beta cell while preserving the immune response to foreign antigens (**Tables 1, 2, 3 and 4**).

Of the agents tested for the prevention of T1D in first degree relatives of T1D patients, oral/inhaled insulin was the most hopeful, but proved disappointing in clinical trials. This treatment strategy aimed at protecting beta cells from the cytotoxic attack of mononuclear cells was based on two potential mechanisms: (1) suppression of beta cell antigen expression associated with endogenous insulin secretion and (2) metabolic rest or "quiescence" of beta cells by administration of exogenous insulin [109]. Rodent studies had demonstrated that insulin given before disease onset was able to delay or even stop

Figure 71. Intervention Opportunities in the Virally Inducible BBDR Model of T1D



progression of beta cell demise [110-112], and these results were initially corroborated by a pilot study in unaffected first degree relatives of T1D patients [113]. However, none of the subsequent clinical trials with oral or parenteral insulin administration were able to demonstrate a protective effect over placebo control [114, 115].

Agents proving to be more successful in clinical trials include those designed to modulate the autoreactive process in new onset diabetics so as to maintain clinically significant levels of remnant endogenous insulin production (measured primarily by C peptide levels and reduction in exogenous insulin requirement over time). Of these agents, the most promising include: DiaPep277 (immunomodulatory humanized peptide from heat shock protein 60, Hsp60), GAD65, and humanized non-FcR binding anti-CD3 monoclonal antibody. The first two of these agents, DiaPep277 and GAD65 are antigen specific agents used to induce immune tolerance to islet antigens, while anti-CD3 mAb is a non-antigen specific immunosuppressant. In two prospective phase II trials, DiaPep277 treatment did not show significant improvement in C peptide levels, but there was a modest trend towards better maintenance of beta cell function compared with placebo [116]. There was no reduction in HbA1c or insulin requirements in the DiaPep277 treated group compared with placebo in these trials [116]. A trial with GAD65 in new onset T1D subjects demonstrated higher fasting C-peptide levels at 30 months in the treatment versus the placebo group, but also did not lower the insulin requirements in the GAD65 treated cohort [117]. These data demonstrate that antigen specific agents offer only modest protection from further beta cell destruction compared with placebo control.

Two different humanized anti-CD3 mAbs are currently in clinical trials in new onset diabetics: teplizumab (hOKT3γ1(Ala-Ala)) and otelixizumab (ChAglyCD3) [118]. A 12 day course of teplizumab or placebo control was given to diabetics within 6 weeks of disease onset [46, 119]. Of the 21 treated with the drug, 15 (71%) maintained or improved C-peptide responses after 1 year compared with 4 of 19 (21%) controls. In the teplizumab treated patients, insulin usage was reduced and HbA1c levels improved during the course of treatment. There were no serious side effects other than a single event of thrombocytopenia which resolved following discontinuation of the drug. A trial with otelixizumab in new onset diabetics demonstrated that at 6, 12 and 18 months following iv treatment with the drug for 6 days, residual beta cell function, measured by C-peptide release, was better maintained in the treatment group than placebo [120]. This effect was most pronounced among patients with initial residual beta cell function at or above the 50^{th} percentile of the treated group. The insulin requirement was also decreased for the otelixizumab group compared with placebo. In trials for both teplizumab and otelixizumab, adverse events were self-limited but included those typical of cytokine release (fever, headache, gastrointestinal symptoms, rash). Transient reactivation of Epstein-Barr virus with signs and symptoms of mononucleosis was observed in the otelixizumab trial. Despite the side effects, the advantage of anti-CD3 therapy over other emerging therapeutics has become apparent from clinical trials: (1) it is a short course of treatment that does not require continuous immunosuppression and (2) it reduces the loss of insulin production over the first 2 years of disease.

The mechanisms of anti-CD3 action in humans are not entirely clear, but those that are not involved are more certain. The mAbs coat and induce moluation of the T cell receptor

but do not eliminate T cells [118]. Although the treatment causes a transient decrease in the number of circulating lymphocytes, by 2 weeks after the last drug dose, the number of circulating T cells rebounds to pretreatment levels [118]. There has been evidence of an increase in the number of circulating CD8+ cells following drug treatment, with these increased levels correlating positively with clinical outcome [121]. These CD8+ cells have further been demonstrated to have regulatory function by virtue of their expression of foxp3 and CTLA-4. The therapy has also been shown to induce the production of the anti-inflammatory cytokine, IL-10 in some individuals [122]. Whether anti-CD3 therapy can induce permanent tolerance to self-antigen will be determined over time.

Therapeutic Potential of Leptin in T1D

If a single emerging therapeutic agent could combine all of the appealing characterics and strategies of antigen-specific therapies (inhaled/oral insulin, DiaPep277 and GAD65) and antigen-nonspecific therapies (teplizumab and otelixizumab), while minimizing each of their side effects, it would be ideal for use at various stages of diabetes. These strategies include: (1) suppressing beta cell antigen expression (2) inducing metabolic rest or "quiescence" of beta cells (3) deleting autoreactive T cells and (4) inducing a tolerogenic T cell population with (5) a short course of treatment that does not require continuous immunosuppression. We demonstrate that leptin treatment at various stages of virally induced T1D combines several of these strategies and may potentially be used as a single therapeutic agent for this disease. Our *in vivo* studies have evaluated leptin as a therapy at multiple stages of T1D. We find that leptin has a protective effect during prediabetes, at diabetes onset, and during islet transplantation. Although leptin treatment has been tested in the prevention of T1D [87] and

in new onset diabetics in the NOD model [102], to our knowledge our study is the first to test the effects of leptin in an autoimmune diabetes recurrence model with islet transplantation.

Hyperleptinemia Induced by AdLeptin and rrLeptin Protects BBDR Rats from pIC + KRV Mediated T1D

The adipokine, leptin, has been demonstrated to inhibit glucose stimulated insulin secretion from beta cells and protect beta cells from lipoapoptosis [98, 99]. The TLR3 ligand, pIC, on the other hand, has been demonstrated in both *in vivo* and *in vitro* experiments to promote beta cell apoptosis [123, 124]. The combination of pIC + KRV induces autoimmune beta cell destruction and T1D in nearly 100% of treated BBDR rats [50]. We hypothesized that the metabolic beta cell cytoprotection afforded by a hyperleptinemic state may be able to overcome the autoimmune beta cell destructive effects of pIC + KRV treatment in the BBDR rat model.

We pretreated 21-25 day old male and female BBDR rats with a single dose of $1x10^{10}$ infectious units/0.5 ml PBS/ 100g body weight of AdLeptin, AdBeta-gal or volume equivalent of PBS and then initiated the pIC + KRV diabetes induction protocol. Diabetes was defined as two consecutive days of high blood glucose levels > 250 mg/dL. Based on these criteria, 91% of the hyperleptinemic, AdLeptin pretreated rats remained diabetes-free during the 45 days of follow-up, compared with 50% in the AdBetagal and 10% in the PBS + pIC + KRV treated controls (**Figure 7**). Histological analysis of pancreatic islets confirmed the protective effects of AdLeptin. The pancreas from AdLeptin + pIC + KRV treated rats that remained non-diabetic at the end of the study demonstrated minimal (1+) to no insulitis

and positive insulin and glucagon staining. Pancreata from diabetic PBS + pIC + KRV and AdBetagal + pIC + KRV treated rats, in contrast, contained involuted, end stage islets with positive glucagon staining, but no insulin positive beta cells (**Figure 14**).

Several metabolic effects of AdLeptin pretreatment in the pIC + KRV model were assessed in our study, including body weights, serum leptin levels, and non-fasting serum insulin levels. AdLeptin pretreated BBDR rats did not gain weight as quickly as rats in other treatment groups, but still exhibited linear growth (**Figures 8 and 9**). Four days following adenovirus treatment, AdLeptin treated rats had serum leptin levels that were 35 times that of the AdBetagal and PBS pretreated groups (**Figure 11**). These serum leptin levels remained elevated by six fold at the end of the study. The hyperleptinemic state was associated with decreased non-fasting serum insulin levels in AdLeptin pretreated rats on both day 0 and day 14 of the study (**Figure 13**). Non-fasting blood glucose levels also remained lower in AdLeptin treated rats during the first two weeks following KRV treatment (**Figure 10**). These observations are in line with previous reports of hyperleptinemia inducing decreased body weight, hypoinsulinemia and decreased peripheral blood glucose concentrations in rodents [125].

Given the reported anorexigenic effects of leptin an alternative explanation for the observed lack of weight gain, concomitant with a decrease in serum insulin and blood glucose levels in the AdLeptin + pIC + KRV treated rats, may be from the lack of nutrient intake in this group, [126]. Food intake was not measured in our study, nor did we have a pair-fed control group, so we cannot rule out the possibility that decreased food intake itself may have prevented pIC + KRV induced diabetes. Additionally, Cusin and colleagues

demonstrated that chronic central leptin infusion (12 μg/day) into lean rats resulted in decreased body weight and serum insulin levels, while increasing insulin-stimulated glucose utilization by muscle and brown adipose tissue [127]. We did not test whether the hyperleptinemia induced by AdLeptin in our model resulted in elevated leptin levels in the CNS of treated rats. Hence, the hypothalamic actions of leptin in protecting the AdLeptin treated rats from pIC + KRV cannot be ruled out.

In this study we demonstrated that a hyperleptinemic state could protect weanling BBDR rats from pIC + KRV induced autoimmune diabetes. Many studies in other autoimmune rodent models and human disease, however, have implicated leptin as a pro-inflammatory and pro-autoimmune adipokine [77, 78, 85-87]. In a recent study exploring the effects of leptin in an autoimmune diabetes model, investigators administered exogenous leptin to newborn NOD mice (the majority of which normally become diabetic between 19-20 weeks of age) and observed a rapid acceleration in time to diabetes onset. Approximately 90% of leptin-treated female mice developed diabetes by the sixth week of age. Additionally, the mice that became diabetic experienced increased severity of disease (23% of leptin treated NOD females developed DKA compared with none among the PBS treated controls) [87]. This effect was gender and time dependent because neither treatment of newborn male NODs nor older female NODs (10 weeks old) with exogenous leptin accelerated diabetes.

In contrast, in our study, pretreatment with either AdLeptin, or continuous treatment with rrLeptin followed by pIC + KRV induction protected between 90-100% of both male and female weanling BBDR rats from T1D. The islets of diabetes-free, leptinized rats in our study were free of insulitis. The apparently contradictory findings between the leptin studies

in the NOD mice and the BBDR rat may be due to (1) a true difference in the physiological effects of leptin in the NOD versus the BBDR (2) differences in age at treatment initiation influencing outcome (3) dose dependent effects of leptin. In our prevention trial with AdLeptin and rrLeptin, we clearly demonstrate that therapeutic intervention with leptin generated serum hyperleptinemia which resulted in many of the reported physiological effects of leptin, namely, lack of weight gain and hypoinsulinemia. The lack of insulitis in the islets of AdLeptin or rrLeptin treated rats may have be from (1) the absence of an autoimmune effector population that can home to the target beta cells (2) insufficient upregulation of chemokines or cell adhesion molecules in the islets to allow autoreactive effectors to home to the target (3) lack of antigen presentation at the target tissue site.

Another recent study in NOD mice has findings that support our results with the AdLeptin trial. Oge and colleagues demonstrated that prenatal undernutrition of pregnant NOD dams had a marked protective effect on the development of T1D in their offspring [90]. Forty percent diet restriction of pregnant NODs from day 12.5 to 18.5 day of gestation resulted in undernournished, low birth weight offspring that had a cumulative incidence of spontaneous diabetes at 24 weeks of 48% among female offspring of diet-restricted dams compared with 73% in normal weight offspring born to non-diet restricted dams. Diet restricted dams had decreased serum leptin levels at the end of the diet restriction period (18.5 days), but their undernourished offspring had significantly elevated leptin levels at 4 weeks compared with normal weight controls. Investigators of this study propose that low birth weight among offspring appeared to exert protective effects via decreased insulitis (at 12 weeks), and decreased beta cell apoptosis (at 20 weeks) in undernourished mice. Certain

results from this study are similar to the observations we made in the AdLeptin + pIC + KRV treated rats. In both studies, serum hyperleptinemia in the newborn mice or weanling rats was associated with low body weight. Additionally, in both studies, decrease in body weight was associated with protection from T1D and insulitis, implying that perhaps nutritional deprivation was preventing the autoimmune response.

One confounding factor in our study with the adenovirus vector was that only 50% of rats in the AdBetagal + pIC + KRV treated group became diabetic, compared with >90% in the PBS group. This finding raised the concern that perhaps the adenovirus was partially skewing the immune system away from the autoreactive process in our model system. We addressed this issue by replacing the adenovirus vector with an alzet pump delivery system for leptin. We then repeated the protection study in female BBDR rats (**Figure 19**), and observed that rrLeptin afforded equivalent protection from pIC + KRV induced diabetes as did the AdLeptin vector delivery system (**Figure 20**). 100% of rrLeptin treated rats were protected from pIC + KRV induced diabetes during the study period, compared with 20% among vehicle treated controls. These results supported our hypothesis that the diabetes protection was afforded by leptin and not from changes induced by the adenovirus delivery system. rrLeptin treated female rats achieved peak serum leptin levels in the range of 50 ng/ml, and maintained these levels until pumps were removal. These hyperleptinemic rats failed to gain weight similar to the AdLeptin pretreated cohort. The concordance of these findings assured us that the recombinant protein was having similar biological effects on tissues as the endogenously expressed leptin delivered by adenovirus vector. Following removal of the pumps, the high serum leptin levels observed in the rrLeptin treated rats

declined rapidly and coincided with an increase in weight gain in the animals, suggesting that the biological effect of the rrLeptin on weight gain did not last beyond its availability in the serum (**Figure 21**).

In the study using rrLeptin, we asked whether rrLeptin merely delayed onset of diabetes beyond the 45 day follow-up period or if the protection was longer lived. To answer this question, we followed a subset of four rats treated with rrLeptin up to 80 days following KRV (with the last pump removal on day 29), and found that none developed diabetes during this time, confirming the longevity of protection. In integrating the lessons from various studies on leptin's role in autoimmune diseases, as well as our own, we deduce that the protective effects of leptin on development of T1D appear to be time and dose dependent, and may be the result of nutritional deprivation. However, the long-lived protection from virally induced T1D provided by leptin treatment in our study necessitates further evaluation of its therapeutic potential for humans.

Leptin Protection Against pIC + KRV Induced Diabetes Correlates with Decreased Splenic Immune Cell Populations

One possible mechanism by which leptin treatment may be protecting from pIC + KRV diabetes is by preventing the generation of the autoimmune effector population. We analyzed lymphocyte and antigen presenting cell (APC) populations in the spleen and lymph nodes of rrLeptin (LPK) and vehicle control (VPK) treated BBDR rats undergoing diabetes induction(**Figure 24**). As early as day 6 post KRV treatment, we observed a decrease in the spleen to body weight ratio and total splenic cellularity in LPK versus VPK treated rats

(**Figures 25,26 and 27**). In fact, the splenic cellularity in the leptin group was not different from the vehicle alone group. This finding implies that the virus induced lymphocytosis observed in the spleen was abrograted in the presence of a hyperleptinemic state.

With regard to different cell frequencies, we observed a modest increase in B and T cell frequencies in the rrLeptin + pIC + KRV (LPK), but no change in frequencies of CD4+ and CD8+ T cell subsets or in DC/MACs. However, because the total cellularity in the spleen was diminished, the total numbers of DC/Macs, B and T cell populations in the spleen were significantly diminished in the rrLeptin + pIC + KRV treated rats compared with vehicle controls.

Subsets of DC/MACs and B cells expressing surface class II MHC with or without the costimulatory molecule, CD86, were not significantly different between any treatment groups. Similarly, T cell subsets including CD4+CD25+, CD4+CD25+Foxp3+ and CD8+CD25+ cells did not change in their frequencies between VPK and LPK treated group. However, the frequency of C8+CD25+ was significantly higher in the spleens of both VPK and LPK treated rats compared with vehicle control treated rats on day +6 (**Figure 32**). These results corroborate earlier findings by Chung and colleagues who reported an increase in the frequency of splenic CD8+CD25+ populations of rats treated with KRV [56]. However, when considering the low splenic cellularity of LPK treated rats, the total number of CD8+CD25+ cells in the LPK group was significantly lower than in VPK treated rats, and approached numbers observed in the vehicle alone treated group (**Figure 33**).

Our data demonstrating a suppressive effect of hyperleptinemia on splenic immune cell populations in the pIC + KRV model does seem to contradict certain studies where exogenous leptin treatment has been reported to promote activation and proliferation of peripheral lymphocytes [80, 81, 83, 128]. Whether the decrease in splenic cellularity we observed is due to lack of cell proliferation or from increased cell death is currently being explored further. We have also considered the possibilities that hyperleptinemia may be (1) having central effects to dampen the immune response or (2) promoting downregulation of the leptin receptor, ObR, on peripheral lymphoid tissues such that the downstream effects of leptin are abolished in those cells. Evidence to suggest central effects of leptin in immunosuppression has been advanced by Okamoto and colleagues [100]. These investigators examined the effects of intracerebroventricular (icv) injection of murine recombinant leptin on the proliferative response of splenic lymphocytes to the mitogen, concanavalin A (conA) in rats, and determined that the conA response of splenic lymphocytes was markedly reduced 30 min after icv injection of leptin. This suppressive effect of leptin was abolished completely either by surgical severing of the splenic nerves or by icv injection of an antibody against corticotropin-releasing hormone (CRH). The results of this study suggest that if icv concentrations of leptin were high (hyperleptinorrachia), peripheral suppression of lymphocyte proliferation may occur, perhaps by leptin induced increase in hypothalamic CRH, which may lead to increased peripheral cortisol levels. In the LPK treated rats in our study, it is possible that serum hyperleptinemia generated high icv levels of leptin which contributed to peripheral immunosuppression.

However, there is also evidence to suggest that high leptin levels can depress lymphocyte proliferative responses independent of its central effects. Investigators have demonstrated that with strong TCR ligation, as is achieved with anti-CD3 and anti-CD28 stimulation, high doses of leptin can have a direct anti-proliferative effect on splenocytes [101]. We performed an *in vitro* stimulation study of BBDR splenocytes, and determined that at high doses of anti-CD3 and anti-CD28, higher doses of rrLeptin (50 ng/ml and 100 ng/ml) had decreased proliferative effects on CD4+ and CD8+ splenocytes 72 hours following stimulation (**Figure 4b**). Because the serum concentrations of rrLeptin in our pIC + KRV treated rats were in the 50 ng/ml range, we hypothesize that strong TCR ligation, perhaps the result of viral antigen presentation by APCs, combined with high serum rrLeptin may have prevented or even suppressed proliferation of many effector T cells. Although the total number of CD4+CD25+ splenocytes in the VPK and LPK treated groups did not differ on day +6, there was a significant decrease in the total number of CD8+CD25+ T cells in the spleens of LPK compared with VPK treated rats. In reviewing the possibilities, we conclude that the exact mechanism by which hyperleptinemia is protecting from T1D in our model most likely involves multiple central and peripheral pathways which require further investigation.

rrLeptin Restores Euglycemia and Prevents DKA in New Onset pIC + KRV Diabetics

Several studies of new onset T1D in humans have reported that metabolic decompensation in children with new onset T1D is associated with dramatic changes of the leptin axis [129-131]. Previous studies have demonstrated that insulin stimulates adipocyte secretion of leptin [132-134]. Hence a state of insulin deficiency may be associated with

decreased serum leptin levels. In fact, leptin levels have been reported to be reduced, particularly in children presenting with severe insulinopenia and DKA [135], but not always in patients who present without DKA [136]. Treatment of acidotic patients with exogenous insulin helps restore serum leptin levels [131, 135], but does not change serum leptin levels in patients who are not in DKA [136, 137]. These studies exploring the leptin-insulin axis in human T1D suggest a complementary role for leptin in glycemic control and ketosis in insulin deficiency states.

A recent study by Yu and colleagues demonstrated that generating serum hyperleptinemia in new onset type 1 diabetic NOD mice using the AdLeptin vector system could restore normoglycemia in the absence of endogenous or exogenous insulin [102]. These investigators hypothesized that leptin may be able to replace the function of insulin in absolute insulin deficiency states. They demonstrated that treatment of diabetic NOD mice with AdLeptin generated serum hyperleptinemia that averaged 319 ± 76 ng/dl three days post treatment, levels that declined rapidly thereafter to 16 ± 4 ng/dl by the ninth day post treatment. In response to this hyperleptinemia, blood glucose levels that had averaged 534 ± 199 mg/dl prior to AdLeptin treatment fell to normal in every AdLeptin treated mouse to 77 ± 67 mg/dl at 9 days post infection. By 22 days, the blood glucose gradually increased to 410 ± 146 mg/dl, corresponding with the decline in serum leptin levels. However, hyperleptinemia was able to render diabetic mice normoglycemic for approximately 13 days. This normalization of blood glucose occurred in the absence of measurable plasma insulin or pancreatic preproinsulin in hyperleptinemic mice. Similar reversion to normoglycemia was not observed in diabetic mice treated with the control vector, AdBetagal. The AdBetagal

treated diabetic mice continued to experience weight loss, hyperglycemia and ketosis. Hyperleptinemic NOD mice, in contrast, decreased food intake to 51% of AdBetagal treated controls, and their weight loss halted ~ 7 days after AdLeptin injection, with an increase in body weight thereafter.

Studies have shown that the insulin deficient state in T1D results in uncontrolled hyperglucagonemia that contributes to the extreme catabolic state resulting in hepatic overproduction of glucose and ketones [138-140]. Yu and colleagues demonstrated that 30 days following AdLeptin or AdBetgal treatment of diabetic NODs, AdBetagal treated controls had significantly elevated serum glucagon levels compared with the AdLeptin treated mice. Furthermore, they demonstrated that glucagon levels in the hyperleptinemic mice were not significantly different from prediabetic mice. As a result of these findings, the investigators concluded that leptin mediated suppression of diabetic hyperglucagonemia contributed to the reversal of the diabetic state among hyperleptinemic mice.

The investigators in this study ruled out the possibility that leptin was potentiating the effects of residual insulin by demonstrating that there was no residual insulin in the pancreas (undetectable preproinsulin mRNA). Additionally, they demonstrated that a hyperleptinemic state could restore normoglycemia in both normal (single dose of 80 mg/kg) and high dose (two doses of 80 mg/kg) streptozodocin (STZ) and alloxan treated (single dose of 100 mg/kg), lean, wild type Zucker diabetic rats. The investigators demonstrated that the mechanisms by which serum hyperleptinemia restored normoglycemia in the high dose STZ diabetic rats was by suppression of glucagon-mediated gluconeogenesis in the liver, and the

potentiation of the insulinomimetic effects of insulin-like growth factors (IGF-1) on skeletal muscle.

In our trial of leptin treatment in new onset diabetics, we observed a significant survival benefit and prevention of DKA with rrLeptin treatment in the absence of exogenous insulin therapy. We treated weanling male and female BBDR rats with pIC + KRV, and upon diabetes onset, treated them with rrLeptin (1.5 µg/g/day) or vehicle delivered by alzet pumps, without any additional exogenous insulin therapy. All rats were treated on the second day of high blood glucose, and followed for up to 20 days post pump insertion until they met at least one of three criteria: (1) body weight loss >20% following pump insertion, (2) urine ketone positivity for 2 consecutive days or (3) moribund appearance. We utilized two different doses of pIC (1 and 2 µg/g body weight) with KRV to render rats diabetic. Rats in the 2 µg/g pIC group had an accelerated time to diabetes onset compared with those in the 1 µg/g pIC group, an effect that may be associated with greater islet damage at the time of diabetes onset in this group.

Two of three diabetic rats in the 1 µg/g pIC experiment, and one of eight in the 2 µg/g pIC experiment experienced restoration of euglycemia with rrLeptin treatment (**Figures 50 and 53**). The duration of this euglycemia was variable, ranging between 6 and 18 days, with one rat in each experiment remaining euglycemic at the end of the study period. Most importantly, rrLeptin was able to prevent DKA in new onset diabetics, as none of the leptin treated rats in either study became urine ketone positive during follow-up. Based on these data, we hypothesize that leptin mediated suppression of glucagon may be preventing excessive ketosis in our model, as was observed in the NOD model [102].

Although we did not observe restoration of euglycemia in as many rrLeptin treated rats in the two pIC + KRV experiments as Yu and colleagues observed in AdLeptin treated NOD diabetics, we also did not achieve the high serum leptin levels using alzet pumps (37 ± 8 ng/ml by 5-6 days post pump insertion) as these investigators achieved with the AdLeptin delivery system (319 ± 76 ng/dl by 3 days post AdLeptin injection) [102]. Perhaps with higher serum leptin levels, we could achieve restorative euglycemia in a greater percentage of the pIC + KRV diabetic rats. We also cannot rule out the possibility in our model that leptin is potentiating the effects of residual insulin, as BBDR rats treated on the second day of high blood glucose still have residual islet mass and serum insulin (unpublished data). The difference in residual insulin levels may explain the variation in duration of restorative euglycemia observed in our rats. Regardless, evidence from two different species (NOD mice and BBDR rats) and two different T1D disease models (spontaneous and virally inducible) provides proof that leptin treatment can restore euglycemia in the absence of exogenous insulin therapy in new onset diabetics. This effect of leptin, therefore, can be manipulated perhaps to improve glycemic control and reduce the insulin requirement in new onset diabetics.

Effects of rrLeptin on Islet Transplants in pIC + KRV Diabetic Rats

Thus far, we have demonstrated that treatment of BBDR rats with rrLeptin prior to pIC + KRV induction can prevent disease onset and preserve beta cell mass. We have also demonstrated that treatment of new onset diabetics in the pIC + KRV model system with rrLeptin can improve survival, restore normoglycemia in a subset of treated rats, and prevent DKA. We next wanted to determine if hyperleptinemia could enhance islet graft survival in a

transplantation model. Transplantation currently remains a last resort for patients with T1D who suffer from long term micro- and macrovascular complications of the disease or from labile disease [33]. Islet transplantation, an investigational procedure, is considered a less invasive technique than whole pancreas transplantation, the current gold standard for beta cell replacement therapy. Despite the success of the Edmonton protocol in using a new steroid-free immunosuppressive therapy, a combination of sirolimus and low dose calcineurin inhibitor (tacrolimus), and induction with an anti-IL2-receptor antibody (daclizumab), only 44% of transplant recipients are reported to maintain insulin independence and adequate glycemic control 1 year after transplantation, 31% at 2 years, and a dismal 10% at five-years post-transplant [35]. The potential causes of failure of islet transplants have included failure of initial engraftment, inflammatory response at the transplant site, alloreactive (rejection) or autoimmune response, and beta cell toxicity [141].

Lee and colleagues have shown that the failure of beta-cells in islets transplanted via the portal vein into the liver may be caused by excess insulin-stimulated lipogenesis and lipotoxicity to the graft [142]. They hypothesized that reducing lipogenesis by leptin or caloric restriction could prevent or reduce the destruction of transplanted beta cells. To test this hypothesis they transplanted syngeneic islets via the portal vein into the livers of STZ treated diabetic rats. Four weeks after transplantation, as islet grafts began to fail, the investigators found elevations in the mRNA of the lipogenic transcription factor, sterol regulatory element-binding protein-1c (SREBP-1c), its lipogenic target enzymes, and liver triacylglycerol (TG) content. Hepatocytes surrounding the islets with damaged beta cells were positive for oil red O staining for lipids and immunostaining for SREBP-1. When the

investigators combined islet transplantation with AdLeptin treatment (generating serum hyperleptinemia) or caloric restriction, they found that leptin-induced lipopenia prevented, and caloric restriction reduced steatosis, hyperglycemia, and apoptotic beta-cell destruction in the graft. Their results suggested that antilipogenic effects of a hyperleptinemic state might improve outcomes following islet transplantation.

The dual lipolytic and immunosuppressive effects of leptin prompted us to test the hypothesis that hyperleptinemia could protect islet grafts transplanted into pIC + KRV induced diabetics from both metabolic stress and autoimmune recurrence. We transplanted male and female diabetic BBDR rats (rendered diabetic with pIC + KRV) with 10 syngeneic islets/g body weight under the kidney capsule, and implanted an alzet pump with rrLeptin or vehicle control. Ten days later, the pumps were replaced, and the second pump was removed after 2 weeks (**Figure 54**). We found that all three islet transplanted rats in the rrLeptin treated group remained normoglycemic following transplantation until the pumps were removed (day 24 post transplant), after which they reverted to hyperglycemia. Three of four vehicle control treated rats became hyperglycemic during the follow-up period. Median diabetes survival in this group was 17.5 days compared with 30 days in the rrLeptin treated group. However, the delay in diabetes-free survival afforded by rrLeptin was not statistically significant.

Since rrLeptin treated rats became hyperglycemic soon after the pumps were removed, we surmised that enhancement of islet graft survival may be dependent upon continuous treatment with leptin. Therefore, we modified our islet transplantation protocol to

include up to four pump replacements following the first insertion during a 50 day follow-up period (**Figure 58**). Due to the extensive surgical manipulations associated with multiple pump replacements, we excluded several animals that developed infectious seromas or pump-site ulcers from the study. Of the two remaining animals treated with rrLeptin, both remained non-diabetic until the last day of the study when one reverted to hyperglycemia. In the vehicle control group, 3 of 6 became diabetic during the study, with a median diabetes free survival of 27.5 days. The difference in diabetes free survival was not significant between the two groups, perhaps owing to the small sample size in this experiment. Pooled data analysis of the 20 day diabetes-free survival between groups in both these studies did demonstrate a survival benefit of rrLeptin over vehicle, but this result was borderline significant (**Figure 62, p=0.07**). Of concern to us from the alzet pump studies was that only 50% of rats in the vehicle control group experienced autoimmune recurrence of diabetes. We hypothesized that this may be due to either the lack of a robust reactivation of the autoimmune response following islet transplantation in the pIC + KRV diabetes model or the potential skewing of the autoreactive process by surgical manipulation.

AdLeptin Significantly Protects Islet Transplants in pIC + KRV Diabetic Rats

To avoid surgical manipulation, we used the AdLeptin vector system to achieve serum hyperleptinemia in the islet transplantation protocol (**Figure 63**). Male and female rats were rendered diabetic with pIC + KRV, and administered i.v. AdLeptin or AdBetagal four to five days prior to transplant to allow for gene expression. Diabetic rats were treated with 10 islets/g body weight of syngeneic islets under the kidney capsule, and monitored for up to 50 days post transplant for recurrence of diabetes.

In this protocol, we observed a significant difference in the diabetes free survival of AdLeptin treated rats compared with AdBetagal controls (**Figure 64**). The diabetes free survival in the AdLeptin treated rats was 90%, compared with 30% in the AdBetagal group. Median survival for AdLeptin treated rats was 50 days, compared with 26 days for AdBetagal treated rats. Serum leptin levels in AdLeptin treated rats went from 0.8 ± 0.4 ng/ml prior to adenovirus treatment to 37 ± 12.4 ng/ml immediately prior to transplantation while among AdBetagal controls leptin levels remained low at 1.4 ± 0.5 ng/ml. In the study by Lee and colleagues, hyperleptinemia in the context of islet transplantation prevented body weight gain while maintaining euglycemia [142]. Similarly, AdLeptin treated male and female, islet transplanted rats failed to gain weight during the study (**Figure 65**), and remained normoglycemic during the study, occasionally becoming hypoglycemic (**Figure 66**). All AdLeptin and AdBetagal treated rats that remained normoglycemic at the end of the study reverted to hyperglycemia following uninephrectomy of the islet bearing kidney. This reversion indicated that the islet bearing graft and not residual endogenous islets contributed to the euglycemia in these rats.

Histological analysis of the islet grafts from all non-diabetic AdLeptin treated rats demonstrated that grafts stained positively for both insulin and glucagon. However, we did observe large lymphocytic infiltrates surrounding the grafts of rats that remained diabetes free. This infiltrate did not appear to be a destructive insulitis as beta cells in the graft still had extensive positive staining for insulin (**Figure 67**). In contrast, islet grafts from diabetic rats in the AdBetagal group showed glucagon but not insulin positivity, and had little to no remnant immune infiltrate surrounding the graft (**Figure 70**). We cannot exclude the

possibility, that with longer follow-up, the immune infiltrates surrounding islet grafts of AdLeptin rats may have been able to destroy the beta cells. We would then need to consider the serum leptin levels that are associated with progression to disease in this group. However, we deduce from our histological findings that the lymphocytic infiltrate surrounding the islets of AdLeptin treated, non-diabetic rats most likely included memory cells that were able to home to the site of the transplanted islets, but were unable or less efficient in killing their targets. Whether this effect was due to a decrease in class I MHC expression by the grafted beta cells rendered quiescent by leptin, or from increased infiltration of Tregs or decreased effector cell function with rrLeptin treatment remains to be elucidated.

The effect of leptin on insulitis in the diabetes recurrence model is in contrast to that observed our prevention studies where there was minimal or no insulitis. We conclude from these observations that hyperleptinemia can perhaps inhibit both a naïve immune response as well as primed memory response to autoantigens. The therapeutic potential for leptin in the prevention and treatment of T1D has become apparent from studies presented in this report, as well as those by other investigators. A positive treatment outcome with leptin therapy, however, may critically depend on its dose, timing and delivery. Further studies are therefore warranted in our model and others to dissect the immune and metabolic effects of leptin on target tissues, particularly beta cells.

Future Directions

Several variables in our protection studies merit further investigation. First, we need to investigate the confounding effects of leptin induced low body weight in promoting a

potentially immunosuppressive, starvation state in our rats. This starvation induced immunosuppression may explain the lack of an autoreactive response against beta cells in our model. We can address this confounding variable by including experimental groups in our studies that are pair fed to the AdLeptin treated group and treated with pIC + KRV. If there is no difference in the incidence of T1D in this group, relative to rats treated with pIC + KRV, then we can conclude that the effects of leptin on the autoreactive process are independent of body weight or nutritional status of the host. Second, in the diabetes protection model, we need to further analyze the effects of hyperleptinemia on beta cell function. Although we hypothesized that rrLeptin was driving beta cells into a state of relative quiescence in our pIC + KRV model, we would need to demonstrate this by demonstrating measuring glucose stimulated insulin secretion or decreased class I expression on these beta cells. Third, the effect of hyperleptinemia on the functional capacity of lymphocytes in pIC + KRV treated rats deserves particular attention. We demonstrated that hyperleptinemia promoted immunosuppression by decreasing the total number of lymphocytes and APCs. However, whether these rrLeptin treated cells retain their functional capacity must be determined by performing additional studies, such as adoptive transfer. Failure of splenocytes from leptin treated rats to transfer T1D to susceptible hosts (eg. WAG nude rats), would provide additional proof of the suppressed autoreactive capacity of the lymphocytes exposed to leptin.

We demonstrated that leptin therapy in new onset diabetics could reverse hyperglycemia and prevent ketosis that is commensurate with an insulin deficient state. To understand the mechanism for leptin-mediated glycemic control and inhibition of ketosis in

our pIC + KRV model, further studies are needed. For example, if the reduction in hyperglycemia is due to leptin's suppression of hyperglucagonemia can be determined by measuring serum glucagon levels before and after leptin therapy in new onset diabetics. Leptin's ability to enhance peripheral glucose utilization by skeletal muscle and brown fat can be investigated by measuring glut-4 expression or changes in glycolytic enzymes in peripheral tissues of leptin treated rats.

Hyperleptinemia generated by AdLeptin was also effective in preventing autoimmune recurrence in transplanted islet grafts. Future studies must address whether this protective effect of leptin is from its actions on the transplanted islet grafts, and/or the memory immune cells or Tregs in the grafts of transplanted rats. For example, if treatment of leptin protected islet graft recipients with a Treg depleting antibody (anti-RT6.1) decreases islet survival in otherwise protected rats, then we can deduce that leptin's protective effects on islet grafts may be mediated by its effects on Tregs.

Leptin's pleotropic role in the body, particularly in energy metabolism and immune regulation, makes it an intriguing candidate for further evaluation. However, we must recognize that because it affects multiple organ systems in the body, its therapeutic index may be narrow and its side effect profile, wide. Careful evaluation of its side effect profile in each type of experimental setting is warranted. However, its powerful ability to prevent autoimmune T1D, reverse new onset disease and protect transplanted islets in the pIC + KRV model gives us great hope that this hormone will definitely have a place in the future therapeutic arsenal for T1D.

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