LIPOSOMAL ALPHA-GALACTOSYLCERAMIDE PLUS INSULIN EFFECTS
ON TYPE 1 DIABETES IN NON-OBESE DIABETIC MICE

by

Jason Ken-Shun Hung

BSc. University of California, Davis

A THESIS SUBMITTED IN PARTIAL FUFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate and Postdoctoral Studies
(Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2014

© Jason Ken-Shun Hung, 2014
Abstract

Type 1 diabetes (T1D) is a currently incurable autoimmune disease that affects roughly 35 million individuals worldwide and is caused by impaired glucose homeostasis due to the destruction of insulin-secreting β cells through a breakdown in immunological tolerance to β cell antigens. Several components involved in regulating and suppressing T cell activation by self-antigens are believed to be involved, including regulatory T cells (Tregs), dendritic cells (DC) and natural killer T (NKT) cells. To determine whether regulatory function of these cells can be restored in a model of T1D, we have assessed the preventive potential of a novel liposome that incorporates both the NKT agonist alpha-galactosylceramide (αGalCer) and insulin, a key early antigen involved in the disease pathogenesis. We hypothesized that stepwise activation of the DC, Treg and NKT pathways using this novel agent would prevent diabetes in NOD mice. Liposomal therapy or control agents were administered to NOD mice through different routes from week 4 to week 9 and mice were followed for hyperglycemia. We show that liposomal therapy prevents T1D in non-obese diabetic (NOD) mice compared to untreated mice and that the route of injection was paramount for efficacy. Intravenous injections provided no protection, intraperitoneal injections only delayed T1D, while subcutaneous injections fully protected mice from disease. Interestingly, there was no difference in insulitis scores between treatment groups despite disease outcome. We also show that αGalCer presentation occurs in macrophages and dendritic cells present in the parametrial fat at 24 hours and 72 hours. We conclude that subcutaneous
liposomal therapy using αGalCer and insulin can prevent the development of T1D in NOD mice. We propose that the liposome composition as well as the route of injection alters the pharmokinetics and pharmodynamics of the therapy, possibly by increasing drug stability or by creating drug reservoirs in key organs.
Preface

This dissertation is original and none of the text has been taken directly from previously published articles. All animal experiments were authorized by the Faculty of Medicine and animal care services at the University of British Columbia in conjunction with the Canadian Council on Animal Care (UBC Office of Research Services protocols A10-0006 and A13-0163). All liposomal therapies were produced by REGiMMUNE Inc.

Experiments in Chapter 3 involving NRP-V7/Insulin B\textsubscript{15-23} tetramer staining were performed together by Dr. I-Fang Lee and myself. Experiments involving histological staining and blood glucose level testing were performed with Ms. Nicole J. Leung under my supervision. Islet isolation and tetramer staining were performed together with Dr. Derek L. Lai and Mr. Mitsu Komba. All experiments in Chapter 4 were performed together by Ms. Nicole J. Leung and myself.
Table of Contents

Abstract........................................................................................................................................................................ ii
Preface ........................................................................................................................................................................ iv
Table of Contents .............................................................................................................................................................. ii
List of Tables .................................................................................................................................................................... v
List of Figures .................................................................................................................................................................. vi
Acknowledgements ............................................................................................................................................................ viii
Dedication ........................................................................................................................................................................ ix

1 Introduction .................................................................................................................................................................. 1
  1.1 Diabetes Mellitus ...................................................................................................................................................... 1
  1.2 Demographics .......................................................................................................................................................... 2
  1.3 Potential Environmental Triggers of T1D .......................................................... 2
  1.4 Genetics of T1D ...................................................................................................................................................... 3
  1.5 Symptoms and Complications of T1D ................................................................................................................ 4
  1.6 Pathology of T1D ................................................................................................................................................... 5
  1.7 The Pancreas and Islets of Langerhans ................................................................................................................ 6
  1.8 Immunoregulatory Cells: NKT, Treg and DC ...................................................................................................... 7
  1.9 The Non Obese Diabetic (NOD) Mouse Model .................................................................................................. 10
  1.10 Potential Autoantigens in T1D ........................................................................................................................... 11
  1.11 Current Therapies for T1D .................................................................................................................................. 13
4.3 Single Injection of Liposomal $\alpha$Galcer Expands APCs .................. 40

4.4 $\alpha$Galcer Presentation on APCs in Parametrial Fat.................... 42

5 Discussion and Conclusion ........................................................................ 46

5.1 Liposomal $\alpha$Galcer + Insulin Protects Mice From T1D ................... 46

5.2 Summary .................................................................................................. 50

References ..................................................................................................... 53
List of Tables

Table 1 Range of survival curves and hazard ratio values for liposomal αGalCer + insulin therapies…………………………………………..27
List of Figures

Figure 1  Histological staining of islets of Langerhans.........................6

Figure 2  Formulation of liposomal αGalCer + insulin..........................16

Figure 3  Proposed mechanism of action for protection from T1D using
liposomal αGalCer + insulin.....................................................19

Figure 4  Protection of NOD mice injected with αGalCer + insulin from
T1..............................................................................................25

Figure 5  Proportion of NRP-V7+ CD8+ T cells in the blood injected with
liposomal αGalCer + insulin.....................................................29&30

Figure 6  NRP-V7+ CD8+ T cell levels in islets........................................32

Figure 7  Infiltration of islets after treatment with liposomal αGalCer +
inulin.......................................................................................34

Figure 8  Expansion of dendritic cells after liposomal αGalCer + insulin
therapy......................................................................................37

Figure 9  Decreased population of immune cells in the spleen after liposomal
αGalCer + insulin therapy.........................................................39

Figure 10 Expansion of APCs after single injection of liposomal αGalCer....41
Figure 11  αGalCer:CD1d presentation on APCs in the stromal vascular fraction of epididymal adipose tissue..........................44
Acknowledgements

I would like to thank my supervisor Dr. Rusung Tan, Dr. John Priatel and my colleagues in the Tan Lab for all the help and advice they have given me throughout my Master’s program. I would also like to thank my supervisory committee for all their kind suggestions and advice. I am also deeply indebted to Dr. Omar Duramad and Mr. Haruhiko Morita at REGiMMUNE for allowing my participation in their study as well as laying much of the groundwork for this project.

Special thanks are owed to my parents, who were always there and still are with their physical and emotional support.
Dedication

To Lillian
1 Introduction

1.1 Diabetes Mellitus

Diabetes mellitus is a metabolic disease in which the loss of the peptide hormone insulin renders the body incapable of using glucose, its main source of energy. DM is one of the oldest known human diseases and was one of the first ever to be described; etymologically diabetes comes from ancient Greek meaning “to pass through” while mellitus is classic Latin for “honey-sweet”, two words that describe one of the primary symptoms and signs of diabetes, polyuria (excessive urination) and glycosuria (glucose in the urine) (Engelhardt 1989).

In healthy individuals, insulin is produced by β-cells located in the islets of Langerhans in the pancreas in response to high blood glucose levels, which normally occur after eating. Insulin itself acts as a messenger that allows other cells in the body, such as muscle and brain cells, to transport glucose from the blood, which is then converted to energy. While there are two main variants of DM, both of which are caused by insulin dysfunction leading to hyperglycemia (high blood sugar); type 1 diabetes (T1D, previously known as juvenile diabetes) is an autoimmune disease caused when immune cells improperly attack an individual’s own β-cells while type 2 diabetes (T2D, previously known as adult-onset diabetes) is caused when the body becomes resistant to insulin signaling as well as deficient in insulin secretion.
1.2 Demographics

Around 380 million people worldwide have Diabetes Mellitus for reasons largely still unclear. The number of children diagnosed specifically with T1D is steadily rising at about 3% yearly and diabetes as a whole is projected to be the 7th leading cause of death by 2030 with an estimated 3.4 million people dying annually from diabetes related complications (Onkamo et al. 1999; World Health Organization 2013). At home, Canadians can expect to see the number of people living with diabetes rise from 1.3 million in 2000 to 2.5 million in 2010 and 3.7 million by 2020. Economically, the burden of diabetes to Canadian society of all forms of Diabetes Mellitus is expected to reach $16.9 billion dollars annually by 2020 through direct hospitalization cost, the price of therapeutic drugs and the loss of productivity due to diabetes related mortality as well as long-term disability (Doucet & Beatty 2010). Some data suggest there is polar-equatorial gradient with T1D incidence, where countries closer to the poles have slightly higher rates of T1D, possibly through less vitamin D exposure (Mohr et al. 2008) although this is controversial as there is also some evidence that contradicts these findings (LaPorte et al. 1985; Onkamo et al. 1999).

1.3 Potential Environmental Triggers of T1D

While the exact mechanism behind the pathogenesis of T1D is currently unknown, several proposed triggers have been hypothesized. Another possibility is laid out by the hygiene hypothesis, which argues that lack of tolerizing environmental exposure in early childhood leads to a abnormal development of the immune system, which leads to an increase in autoimmune diseases such as
T1D (Singh 2002). On the other hand, the accelerator hypothesis argues that rapidly a increasing population of obese and insulin resistant children leads to borderline insulin deficiency, which increases the risk of developing T1D (Wilkin 2001). Finally, several environmental factors have been proposed that predispose or trigger T1D in patients such as immunizations to other diseases, viral infections (especially the Coxsackie B4 virus), early exposure to cow’s milk (Gerstein 1994) and a reduction of breast feeding, vitamin D and nitrate consumption and early exposure to root vegetables or toxic contaminants (Levy 2011).

1.4 Genetics of T1D

Genetics is the process of inheriting genes from parents to offspring and play a large role in the genotype and phenotype of an individual. While usually associated with physical traits like height or eye color, genetics also can be an important factor in the propensity of an individual’s susceptibility to diseases and autoimmunity. Genetics plays some role in T1D development, although the full extent is currently unknown; broadly the incidence of T1D is about 43 times more likely to develop in children of Caucasian/European decent than in children of Asian decent (Onkamo et al. 1999). While a multitude of genes have been associated with type I diabetes via gene wide association studies (Reddy et al. 2011), tissue typing molecules encoded by HLA Class I and II genes has also shown to be greatly correlated with T1D pathogenesis (Noble et al. 1996; Nejentsev et al. 2007). However only 50% of genetically identical twins whose parents have T1D ultimately themselves become type I diabetic, with two
strongest causes being the HLA-DQ and HLA-DR alleles as well as the insulin gene itself (Jiang et al. 2005; Wucherpfennig & Eisenbarth 2001; Hong et al. 2001; Van Kaer 2005). Combining predominantly Caucasian population and Canada’s proximity to the North Pole is perhaps a good start in explaining why it has the 3rd highest incidence of T1D among its peer countries (Canadian Diabetes Association 2009).

1.5 Symptoms and Complications of T1D

The most common symptoms of untreated T1D include polydipsia (excessive thirst), polyuria (excessive urination), polyphagia (excessive hunger) and weight loss; however patients are usually admitted for diabetic ketoacidosis and often presenting with moderate to severe symptoms such as nausea, vomiting dehydration and lethargy (Cooke & Plotnick 2008). Prolonged and uncontrolled hyperglycemia also comes with its own set of complications, notably microvascular damage that can affect nearly any organ system due to the damaging effects of high levels of glucose on small blood vessels and nerves. Retinopathy is perhaps the most common complication with over 80% of T1D patients who have been diabetic for more than 10 years having decreased vision or blindness (Levy 2011). Ultimately, type I diabetic patients often succumb to cardiovascular disease or diabetic nephropathy, which are again linked to chronic hyperglycemia (Morrish et al. 2001). On the other end of the spectrum, hypoglycemia due to a relative excess of exogenous insulin administration and in serious cases can lead to diabetic coma if not properly monitored (Bending et al. 1985).
1.6 Pathology of T1D

Regardless of the exact trigger for T1D, in the end destruction of β cells is thought to be a primarily T cell mediated process. In the conventional model of T1D immunopathogenesis, β cell antigens are initially captured and processed by antigen presenting cells (APCs) such as dendritic cells (DC), macrophages (Mφ) and B cells, then presented to diabetogenic T cells in the pancreas or local lymph nodes. Helper CD4+ T cells in turn secrete cytokines to recruit and activate cytotoxic CD8+ T cells, which ultimately leads to the induction of β cell apoptosis by the release of granzymes, perforin and other mechanisms like Fas (Yoon & Jun 2006). These diabetogenic T cells then acquire a effector-memory phenotype while still in the pancreas and migrate to peripheral lymphoid tissues to perpetuate the disease (Chee et al. 2014). Diabetogenic T cells are likely present in all individuals, but immunoregulatory cells including tolerogenic dendritic cells, regulatory T cells (Treg) and natural killer T cells (NKT) modulate this autoimmune response in healthy individuals; however there is evidence showing that in diabetic mouse models dysfunction in these immunoregulatory subsets allow the β cells to be destroyed (Boudaly et al. 2002; Tritt et al. 2007; Chen et al. 2007). Clinical presentation of T1D is highly variable ranging from several days to several years in humans meaning that the rate of β cell loss is also highly variable, however it is believed that around 80% of β cells must be destroyed before glycemic control is significantly impaired and most T1D patients still maintain a small population of β cells even after disease onset (Cooke & Plotnick 2008).
Figure 1 Histological staining of islet of Langerhans in NOD mice. Islet in the pancreas histologically stained with hematoxylin and eosin. Islet on the left is from a healthy control while islet of the right is from a diabetic mouse that has been infiltrated by lymphocytes.

1.7 The Pancreas and Islets of Langerhans

The pancreas is an endocrine organ that secretes hormones into the blood stream that regulates metabolism. Moreover, the pancreas is also an exocrine gland that secretes digestive enzymes into the small intestines to aid in the
digestion and absorption of nutrients. The main endocrine structures of the pancreas are the Islets of Langerhans (Figure 1), which are small groups of cells about 200 µm in diameter surrounded by a thin fibrous tissue capsule. Islets of Langerhans are comprised of 4 different cell types: glucagon producing α cells (20% of total islet mass), insulin producing β cells (70%), somatostatin producing δ cells (4%) and pancreatic polypeptide producing γ cells (Wang et al. 2001).

The main function of these cells is to regulate blood glucose levels metabolism as a whole; insulin is secreted to signal the body to absorb glucose from the bloodstream while glucagon does the opposite and signals the liver to convert glycogen into glucose and secrete it into the bloodstream. Somatostatin can block the secretion of both insulin and glucagon (Alberti et al. 1973; Wahren & Felig 1976) while pancreatic polypeptide has been shown to reduce appetite and food intake in humans (Batterham et al. 2003).

1.8 Immunoregulatory Cells: NKT, Treg and DC

NKT cells are a small subset of T cells that share some phenotypic markers of natural killer (NK) cells are have been described by their ability to secrete large amounts of cytokines including interleukin-4 (IL-4) interleukin-10 (IL-10), interleukin 17 (IL-17), interferon gamma (IFNγ) and tumor necrosis factor (TNF) (Rossjohn et al. 2012). A smaller subset of these cells are named invariant NKT (iNKT) cells for their semi-invariant (highly conserved) T cell receptor (TCR), are composed of Vα14-Jα18 α chain and Vβ 8.2/7/2 B chain (Lantz & Bendelac 1994) and by their activation by the non-classical MHC I molecule CD1d (Bendelac et al. 1995), both of which are essential for the selection and function
of iNKT cells (Mendiratta et al. 1997). iNKT cells compose only 0.5% of the total T cell population in mice and typically only around 1% of the total T cell population in the spleen and lymph nodes (Hammond et al. 2001) but can be nearly 20% of all T cells in the liver (Eberl et al. 1999), their main depot. Despite their relatively small numbers, iNKT cells can exert a profound effect on the immune system, as they have been implicated in protection from fungal infections (Albacker et al. 2013), asthma (Meyer et al. 2008), experimental autoimmune encephalomyelitis (EAE)(Mars et al. 2008), and graft vs. host disease (GvHD) (Hashimoto et al. 2005). An interesting paradox is their ability to promote a diverse range of immune responses by altering the cytokine milieu, from activating T helper 1 (Th1) cell via IFN$\gamma$ or T helper 2 (Th2) cell via IL-4. Due to this dichotomy of function, iNKT cells have been studied both in the context of killing cancer cells as well as suppression of the immune system in autoimmunity.

Regulatory T cells (Tregs) are another distinct subset of T cells previously called suppressor T cells whose primary responsibility is preventing autoimmunity by eliminating or containing self-reactive T and B cells in the periphery and thus maintaining the body’s tolerance to self-antigens. Regulatory T cells are mainly recognized by their expression of transcription factor forkhead box P3 (FOXP3)(Hori et al. 2003) as well as typically expressing both CD4 (a TCR co-receptor) and CD25 (IL-2 receptor subunit $\alpha$)(Sakaguchi et al. 1995). Tregs have multiple mechanisms by which they can induce tolerance, including their ability to secrete granzymes that directly kill autoreactive immune cells (Gondek et al.)
2005) and their ability to secrete suppressive cytokines, namely interleukin-10 (IL-10) and transcription growth factor β (TGFβ), which inhibit effector T cell expansion. Tregs require high levels of interleukin-2 (IL-2) for survival and optimal function and thus can be considered a "IL-2" sink that deprives autoreactive T cells of the IL-2 needed to expand (Pandiyan et al. 2007).

Furthermore, NKT cells can secrete IL-2, suggesting a mechanism by which they may promoting Treg expansion and thus indirectly promote suppression (Li et al. 2008; Jiang et al. 2005). Tregs also compose a small subset of the total T cell population in humans and mice and are estimated to be about 5-10% of peripheral CD4+ T cells (Ahmadzadeh et al. 2008).

Dendritic cells are another specialized subset of immune cells whose primary role is to capture and present antigens to T cells. DCs migrate throughout the body but once activated move to the lymph nodes to present antigenic peptides to effector cells like NKT, Treg, B and T cells thus initiating a specific adaptive immune response. Immature DCs also can become tolerogenic dendritic cells, which facilitate tolerance in the periphery (Gaudreau et al. 2007; Mahnke et al. 2002); however once DCs become activated and mature they lose their tolerogenic and antigen presentation abilities and become essential for the proper development of effector T cells. Dendritic cells typically express the CD11c molecule (integrin α X protein) and MHC class II molecule. Outside of normal antigen presentation via the MHC class II molecule, dendritic cells are also the primary source of CD1d presentation and therefore the also the primary activators of iNKT cells (Spada et al. 2000). While there are many different
subsets of DCs, the largest and most commonly studied are myeloid dendritic cells (mDCs), whose primary role is antigen presentation and secreting the cytokine interleukin-12, which skews T helpers cells into a Th1 bias. Plasmacytoid dendritic cell (pDC) are a smaller subset of DCs which secrete type I interferons and have been implicated in autoimmunity (Moseman et al. 2014; Diana et al. 2013).

1.9 The Non Obese Diabetic (NOD) Mouse Model
T1D is a very difficult disease to predict and diagnose early as patients typically only are discovered to be diabetic after presenting diabetic ketoacidosis at which point most of β cell loss has already occurred. For this reason, several animal models have been developed including the BioBreeding Rat, the Tokushima rat and the non-obese diabetic (NOD) mouse; however the NOD mouse model has become a gold standard for T1D research as it develops spontaneous diabetes including mononuclear cell infiltration into the pancreatic islets leading to an almost complete depletion of insulin (Tarui et al. 1986). About 70-80% of female NOD mice develop spontaneous diabetes typically around 8-16 weeks of age while only 20-30% of males develop diabetes, although peri-insulitis can be found in as short a time as 2 to 3 weeks after birth.

Furthermore NOD mice have multiple developmental and functional defects in their immunoregulatory cells, suggesting similar defects may play a role in the pathogenesis of the disease in humans, which have been implicated as a
possible component to T1D pathogenesis in humans. NOD mice have been shown to contain fewer NKT cells in comparison to C57BL/6 and BALB/c mice (Poulton et al. 2001) but express higher levels of iNKT17 cells (Simoni et al. 2011), a small subset of iNKT cells that secrete interleukin-17 (IL-7), a powerful pro-inflammatory cytokine implicated in promoting multiple autoimmune diseases. NOD mice also have a lower capacity to generate Tregs, with NOD mice typically having about half the Treg population compared to autoimmune resistant mice (Salomon et al. 2000). Finally NOD mice exhibit APC dysregulation including abnormal macrophage development and maturation (Serreze et al. 1993; Piganelli et al. 1998) and differentiation and proliferation abnormalities in dendritic cells (M. Lee et al. 2000).

1.10 Potential Autoantigens in T1D

Multiple candidate antigens have been postulated for T1D in both humans and mice, including insulin (specifically pro-insulin chain B: 9-23), islet-specific glucose-6-phosphatase catalytic subunit-related protein (IRGP\textsubscript{206-214}), glutamic acid decarboxylase 65 (GAD65) chromogranin A (ChgA) and heat shock protein 60 (hsp60) (Nakayama et al. 2005; Ouyang et al. 2006; Kaufman et al. 1993; Stadinski et al. 2010; Elias et al. 1990). However, with so many potential autoantigens, the question becomes are some autoantigens more important that others and are some autoantigens essential to trigger T1D?
Insulin itself is a logical candidate as an essential autoantigen, however studies have also shown that knocking out the Ins2 gene in a dendritic cell subset called medullary thymic epithelial cells which is involved in central tolerance exacerbated diabetes incidence (Fan et al. 2009). More recent data has come out suggesting that Ins1+/− Ins2−/− mice however are protected from type 1 diabetes, meaning that knocking out Ins2 alone is not sufficient to cause disease (Mehran et al. 2012).

Studies have found proinsulin B:9-23, a small peptide chain of proinsulin might be the key “checkpoint” in diabetes; altering the amino acid 16 in pro-insulin B9-23 from tyrosine to alanine resulted in failure to bind to Kd MHC class I molecule on CD4+ and CD8+ T cells, ultimately leading to no development of insulin auto-antibodies, insulitis or T1D (Nakayama et al. 2005). Studies have also shown that while TCR transgenic mice with CD8+ T cells specific for IGRP206-214 (NOD 8.3 mice) develop accelerated T1D, transgenic NOD mice that over express proinsulin 2 in APCs do not have IGRP specific CD8+ T cells and are protected from T1D. Even when these mice are then crossed with NOD 8.3 mice, T1D incidence is dramatically reduced, suggesting that IGRP is less important than insulin itself as a key autoantigen itself (Krishnamurthy et al. 2006; Krishnamurthy et al. 2008).

One proposed model that may explain the relative importance of T1D autoantigens is epitope spreading (also known as an epitope cascade), which postulates that T1D onset is actually chain of events each triggered by a specific set autoantigens. In this model, the first autoantigen that appears is pro-insulin
B: 9-23 (InsB\textsubscript{9-23}), which is the “checkpoint” epitope. When InsB\textsubscript{9-23} specific diabetogenic T cells begin to destroy β cells, more epitopes are released and in turn activate more diabetogenic T cells with different targets like IGRP, GAD56 and hsp60. Finally, other insulin epitopes from the A and B chains become reactive at which point the cumulative damage to the β cells from these epitopes leads to hyperglycemia and onset of T1D (Luo et al. 2010). The rationale for cure then would be to eliminate the cascade at its first step, namely InsB\textsubscript{9-23}, and thus prevent other T1D autoantigens from appearing.

1.11 Current Therapies for T1D

At present there is no cure for T1D, although the discovery of insulin in the 1920s has dramatically altered management of the disease and allowed patients to live relatively normal lives. Exogenous insulin however does not address the main mechanism of T1D, namely the destruction and loss of β cells in the pancreas. Furthermore, while life saving, insulin therapy is cumbersome, especially for young children, as it requires constant monitoring of blood glucose levels as well as multiple injections in order to keep patients euglycemic. Insulin therapy also has the risk of inducing hypoglycemia due to improper administration or inconsistent meals and can lead to coma or death. Finally, regular and frequent monitoring and injection of insulin is often not economically and logistically feasible in less developed countries and a cure is desperately needed.

One of the many possible immunotherapeutic approaches to treat T1D is ironically insulin itself, with the rationale that activating insulin-specific immunoregulatory cells will lead to suppression of insulin autoreactivity and thus
preventing of T1D. Studies have shown that whole insulin can prevent NOD mice from developing diabetes (Karounos et al. 1997), and that proinsulin combined with anti-CD3 antibody can activate Tregs and induce remission of T1D (Bresson et al. 2006). InsB$_{9-23}$ specifically has been shown to protect or control late-stage pre-diabetes in NOD mice through oral, intranasal or subcutaneous administration or DNA encoding of the peptide (Fousteri et al. 2010; Daniel & Wegmann 1996; Urbanek-Ruiz et al. 2001; Liu et al. 2006).

Unfortunately, a large scale clinical trial of over 80,000 first and second degree relatives of type I diabetic patients showed no difference in disease onset between patients given low-dose subcutaneous insulin injections compared to controls (Group D.P.T.-T.I.D.S, 2002).

Another interesting immune altering reagent is alpha-galactosylceramide ($\alpha$GalCer), a glycolipid originally derived from the marine sea sponge Agelas mauritianus (Natori et al. 1994) that was initially developed as a potential anti-cancer compound (Ishikawa et al. 2005; Nakagawa et al. 2001). It was later found to be a very strong activator of NKT cells (Kawano et al. 1997). In 2001, two groups demonstrated that $\alpha$GalCer treated mice were protected from T1D through a CD1d-iNKT mechanism (Hong et al. 2001; Sharif et al. 2001).

Furthermore, iNKT cells that have been activated with $\alpha$GalCer have also been previously shown to protect NOD mice from T1D by upregulating dendritic cells in the pancreatic lymph nodes (Naumov et al. 2001). However, $\alpha$GalCer therapy has proven problematic in the context of both cancer and T1D due to its toxicity in some animal models (Osman et al. 2000), difficulty to dissolve in a carrier
vehicle and the fact that activated iNKT cells can unpredictably produce both a
Th1 and a Th2 cytokine milieu (Arora et al. 2014). Nevertheless, the promise of
αGalCer for the treatment of T1D may be realized if these issues can be solved.

1.12 Liposomal Therapy

One potential new application that could improve the efficacy and tolerability of
T1D drug therapies including both insulin and αGalCer is to their
pharmacological properties by using a spherical synthetic lipid bilayer or
liposome as a carrier. In the last 15 years liposomal therapies have risen in
popularity and in 2012 a dozen liposomal therapies have been approved by the
FDA while over 20 were in clinical trials (Yeh et al. 2011). Liposomes are also a
versatile tool for drug development since many different variants can be
produced with different sizes, lipid formulations, charges, and drug release and
clearance conditions all contributing to a specialized drug carrier that can be fine-
tuned to each specific disease application (Lian & Ho 2001). Furthermore,
liposomes can be made to be undetectable by the immune system(Yokoe et al.
2008) or can be formulated with various surface proteins and carbohydrates that
will enhance their uptake and response by immune cells including
macrophages(Turk et al. 2004) and dendritic cells(van Broekhoven 2004)
Formulation of liposomal αGalCer + insulin. αGalCer is studded into a synthetic lipid bilayer that also encapsulates whole human insulin.

One example of a promising liposomal therapy to be recently approved by the FDA is vincristine sulfate liposome; vincristine was first approved nearly 50 years ago, and has been used as a chemotherapy on a wide range of cancers including Hodgkin’s and non-Hodgkin’s lymphoma, and most importantly acute lymphoblastic leukemia. However, neuropathic symptoms such as bilateral facial nerve palsies and difficulty walking or extreme pain when walking have largely derailed therapy as dosages low enough to avoid side effects is also too low to be effective (Dixit et al. 2012). With the introduction of vincristine sulfate liposome patients are now able to receive double the dosage of the drug without increased risk of side effects(O’Brien et al. 2013).

Incorporating αGalCer into a liposome is an attractive option for two reasons; one of the major complications with αGalCer is its hydrophobicity and thus traditional methods of dissolving αGalCer is in organic solvents such as DMSO, which
naturally come with their own toxicity issues. The unique composition of liposomes, whose lipid composition contains both hydrophilic and hydrophobic regions, makes it possible to create a completely biocompatible (meaning without adverse side effects from the host) carrier by studding αGalCer within the lipid bilayer. Furthermore, since liposomes can be formulated to specifically targeted APCs (van Broekhoven 2004), a lower dosage may be used that produces equal or even greater immune activation compared to non-liposomal, aqueous αGalCer.

Another benefit of liposomal therapy is that it allows for co-delivery of both hydrophobic and hydrophilic therapies in the same carrier (Figure 2). Whole insulin or specific insulin peptides such as InsB<sub>9-23</sub> can be encapsulated into the interior of the liposome, which has distinct advantages since it can directly carry the insulin peptides directly to APCs where they are protected within an endosomal compartment, and its immunological effects would be enhanced, and the receptor-mediated effects of insulin would not be an issue, including hypoglycemia.

1.13 Proposed Mechanism of Action for Liposomal αGalCer + Insulin

Once injected, the proposed mechanism for liposomal αGalCer + insulin is that APCs such as dendritic cells and macrophages would preferentially phagocytose and process both αGalCer and insulin, which would then be presented by on the cell surface by the CD1d molecule and MHC I molecules respectively. Changes
in kinetics, orientation and binding of αGalCer into the groove of CD1d have been shown to induce a Th2-biased cytokine response in NKT cells (Yu et al. 2005; Arora et al. 2014) and incorporation of αGalCer with a liposome has been proposed to mimic some of these changes, thus also inducing a protective Th2 cytokine milieu. Moreover, co-delivery of both αGalCer and whole insulin will likely boost the protective effect of both Tregs and NKT cells by simultaneous co-activation; αGalCer has been shown to increase the CD4⁺CD25⁺ Treg population as well as augment FOXP3 expression while inhibiting CD4⁺CD25⁻ T cells (Li et al. 2008). Finally, activated NKT cells can exert control over dendritic cell differentiation and migration, likely in a tolerogenic manner (Bollyky & Wilson 2004). Overall, we hypothesize that liposomal αGalCer + insulin therapy may allow for virtually non-toxic administration of αGalCer as well also allow for specific targeting of diabetogenic CD8 T cells. We further hypothesize that simultaneous delivery of liposomal αGalCer + insulin will block islet destruction and prevent T1D (Figure 3).
**Figure 3** Proposed mechanism of action for protection from T1D using liposomal α-GalCer + insulin.
2 Methods

2.1 NOD Mice

Female NOD mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were kept in a pathogen-free animal care facility at the Child & Family Research Institute, which is affiliated with the Children’s and Women’s Hospital and University of British Columbia. All experiments involving experimental animals were approved by Faculty of Medicine and the Animal Care Committee at the University of British Columbia in accordance with the guidelines of the Canadian Council on Animal Care. Blood glucose was monitored weekly with two consecutive readings of 25 mmol/L being considered diabetic.

2.2 Administration of Liposomal $\alpha$GalCer + Insulin

Liposomal $\alpha$GalCer + insulin was provided by Dr. Duramad of REGiMMUNE Inc. (San Francisco, CA USA). Female NOD mice were injected with liposomal $\alpha$GalCer + insulin or controls twice weekly from week 4 to week 9 either intravenously via the tail vein, intraperitoneally or subcutaneously via the tail base. Each injection contained 1µg of $\alpha$GalCer and 10µg of whole insulin. For in-vivo mechanism experiments, female NOD mice were injected with liposome containing 1µg of $\alpha$GalCer subcutaneously at the tail base for either 24 or 72 hours before sacrifice.
2.3 Islet Isolation

Islet isolation was performed by clamping the common bile duct of each pancreas and perfused with collagenase type V in Hank’s balanced salt-solution (HBSS). The pancreas was then removed and minced with scissors. Each treatment group was a pool of 4 pancreases in order to obtain sufficient numbers for analysis. Each sample was then inverted gently and incubated at 37°C for 1 hour. Digestion was stopped by washing with cold HBSS and islets were isolated using a pipet and left in R10 media for 24 hour at 37°C in order to allow islet infiltrates to migrate out. Islet infiltrates were then collected and analyzed with flow cytometry.

2.4 Flow Cytometry

Antibodies recognizing αGalCer:CD1d complex (L363), CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7), CD11b (M1/70), CD11c (N418), CD19 (eBio1D3), F4/80 (BM8) and fixable viability dye (eFluor 506) were purchased from eBioscience (San Diego, CA USA). CD317 (927) and B220 (RA3-6B2) were purchased from Biolegend (San Diego, CA USA). MHCII (550554) was purchased from BD (Franklin Lakes, NJ USA). Murine CD1d tetramer loaded with αGalCer was kindly provided by the National Institute of Health Tetramer Core Facility (Emory University) while the NRP-V7 tetramer was conjugated from peptide produced by Kinexus Bioinformatics (Vancouver, BC, Canada) with streptavidin-PE (E4001)
from Sigma-Aldrich (St. Louis, MO, USA). NRP-V7 tetramers were produced in house by Mrs. Xiaoxia Wang.

Single cell suspensions were generated from spleen and lymph nodes by mashing tissue through a filter and then treating with RBC lysis buffer containing NH₄Cl. Blood was collected in EDTA tubes and mononuclear cells and then separated with Ficoll-Paque gradient centrifugation. Adipose tissue associated lymphocytes were isolated first by mincing up parametrial fat with scissors and then incubating with collagenase (type II, Sigma-Aldrich, St. Louis, MO USA) for 1 hour at 37°C for 1 hour and then filtered. All cells were fluorescently labeled with the indicated antibodies in PBS for 30 mins on ice. Tetramer staining was performed for 1 hour at room temperature before all other antibody staining. Data was acquired using the FACSDiva software either on a LSRII or Canto (BD, Franklin Lakes, NJ USA) and analyzed with FlowJo (Treestar, Ashland, OR USA)

2.5 Histological Scoring of Insulitis

Pancreatic tissue was harvested and fixed in zinc-buffered formalin for 4 hours at room temperature and then mounted in paraffin. 5-micron sections were then cut and stained with hematoxylin and eosin. Five sections from each pancreas sample were cut 100 µm apart to avoid double counting islets. Two blinded observers recorded insulitis scores from a range of 0 to 4: 0, no insulitis; 1, peri-insulitis; 2, insulitis covering less than 50% of the islet; 3, insulitis covering more
than 50% of the islet; 4, insulitis covering the entire islet or loss of islet morphology.

2.6 Statistical Analysis

Protection from T1D was measured using the Gehan-Breslow-Wilcoxon survival curve while statistical significance between treatment groups was measured using One Way ANOVA test followed by Tukey’s test
3 Efficacy of Liposomal αGalCer + Insulin

3.1 Subcutaneously Injected Liposomal αGalcer + Insulin Protects NOD Mice from T1D

Female NOD mice were injected twice weekly from week 4 to week 9 with αGalcer + insulin or controls via three different routes of injection in order to test the efficacy of therapy in T1D protection: intravenous (IV), intraperitoneal (IP) and subcutaneous (SC). No treatment mice developed T1D, in agreement with previous studies in our animal unit (Wang et al. 2001; I.-F. Lee et al. 2011). The Gehan-Breslow-Wilcoxon statistical test is a variant of the logrank (Mantel-Cox) test was chosen since it gives more weight to differences in early time points, which is appropriate in this situation considering that insulitis and disease onset occur early during the experiment. Liposomal αGalCer control did show protection up to week 24, at which point T1D onset increased to become not significant from the no treatment control group. This difference from published reports (Hong et al. 2001) can likely be attributed by the lower titer (1 µg vs. 2 µg) of αGalCer being administered as well as the shortened injection regime (week 4 to week 9 vs. week 4 to week 30). Liposomal insulin control trended slightly towards protection up until week 18, but overall provided no protection compared to no treatment group.
Figure 4 Protection of NOD mice injected with αGalCer + insulin from T1D.

Female NOD mice (N=8) were injected with liposomal αGalCer + insulin or
controls. Blood glucose was monitored weekly with any animal reading over 25 mmol/L twice consecutively being considered diabetic. a Survival curves for no treatment and control therapies. b Survival curves for no treatment and liposomal αGalCer + insulin injected via 3 different routes (IV, IP, SC) of injection. Mice injected subcutaneously were protected from T1D.

Interestingly, route of injection was paramount to the protection from T1D; mice injected with liposomal αGalCer + insulin (IV) showed essentially identical survival curves as no treatment mice while mice injected with liposomal αGalCer + insulin (IP) show protection up to week 27 at which point they rapidly became diabetic (Figure 4).

Only liposomal αGalCer + insulin (SC) injections statistically showed protection from T1D (p=0.044) while liposomal αGalCer + insulin (IP) only trended towards protection but was not statistically significant (Table 1). Hazard ratio is another method of assessing survival and is defined as the slope of the survival curve; for instance the no treatment group had a hazard ratio of 4.225 compared to liposomal αGalCer + insulin meaning that the no treatment group was 422.5% more likely to become diabetic than the group injected with liposomal αGalCer + insulin injected subcutaneously.
<table>
<thead>
<tr>
<th>Comparison</th>
<th>Gehan-Breslow-Wilcoxon</th>
<th>Hazard Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Treatment vs. αGalCer + insulin (SC)</td>
<td>0.044*</td>
<td>4.225</td>
</tr>
<tr>
<td>No Treatment vs. αGalCer + insulin (IV)</td>
<td>0.8573</td>
<td>1.149</td>
</tr>
<tr>
<td>No Treatment vs. αGalCer + insulin (IP)</td>
<td>0.2082</td>
<td>1.573</td>
</tr>
<tr>
<td>No Treatment vs. Insulin only (IP)</td>
<td>0.3482</td>
<td>1.149</td>
</tr>
<tr>
<td>No Treatment vs. αGalCer only (IP)</td>
<td>0.0749</td>
<td>2.939</td>
</tr>
<tr>
<td>αGalCer vs. αGalCer + insulin (SC)</td>
<td>0.8988</td>
<td>1.386</td>
</tr>
<tr>
<td>αGalCer + insulin (IP) vs. αGalCer + insulin (SC)</td>
<td>0.2730</td>
<td>2.687</td>
</tr>
</tbody>
</table>

**Table 1** Range of survival curves and hazard ratio values for liposomal αGalCer + insulin therapies

Overall, liposomal αGalCer + insulin can protect NOD mice from T1D while liposomal αGalCer and liposomal insulin controls did not. However, route of injection drastically changed the rate of T1D onset, with IV injections being essentially the same as no treatment, IP injections only delaying the onset while SC was the only treatment group that protected NOD mice from T1D.
3.2 Liposomal αGalCer + Insulin Decreases NRP-V7⁺ CD8⁺ T Cell Population at Early Time Points in the Blood.

IGRP\textsubscript{206-214} has been implicated as a potential self-antigen in T1D pathogenesis, and is a significant component of early T cell infiltration in islets in NOD mice (Han et al. 2005). Studies have also shown that over-expressing IGRP in antigen presenting cells results in NOD mice having no IGRP specific CD8⁺ T cells, thus leading to protection from T1D (Krishnamurthy et al. 2012). However antibody staining of this epitope is very difficult due to its weak binding affinity so instead the mimotope NRP-V7 (a heteroclitic analogue of NRP-A7) has been extensively used as a surrogate marker for IGRP⁺ CD8⁺ T cells (Lieberman et al. 2003; Amrani et al. 2000). PBMCs were isolated weekly in each group and tetramer stained for the presence of NRP-V7⁺ CD8⁺ T cells. Mice injected with liposomal αGalCer + insulin had lower levels of NRP-V7⁺ CD8⁺ T cells at early time points, especially at week 8 (Figure 5).
Figure 5 Proportion of NRP-V7+ CD8+ T cells in the blood injected with liposomal αGalCer + insulin. a-b Female NOD mice (N=8) were injected with liposomal αGalCer + insulin or controls. PBMC was isolated from week 4 to week 12 and stained with NRP-V7 tetramer. c Percent of NRP-V7+ CD8+ T cells at week 8 (N=8); error bars are representative of the minimum and maximum values recorded.

Interestingly, NRP-V7+ CD8+ T cell percentage did not predict protection from T1D; while all three routes of injections had significantly lower levels of NRP-V7+ CD8+ T cells. Subcutaneous and intravenous injections showed similar
reductions in NRP-V7⁺ CD8⁺ T cell percentages but as mentioned previously only the subcutaneous injections protected mice from T1D.

Finally, InsB₁₅₋₂₃ tetramer staining was done in tandem with NRP-V7 tetramer staining in the blood but showed no significant difference between any treatment group at any time.

3.4 Proportion of NRP-V7+ CD8+ T Cells In Islets Does Not Change with Liposomal αGalcer + Insulin

In conjunction with NRP-V7 tetramer staining in the blood, pancreatic islets were also harvested at week 8 and stained for the presence of NRP-V7+ CD8+ T cells. Measuring NRP-V7+ cells from islet infiltrates is considered a stronger indication of diabetes progression and onset as NOD mice can develop T1D with consistently low levels of NRP-V7+ CD8+ T cells in the blood (Trudeau et al. 2003). Results show that there is no marked difference between any of the treatment groups and levels of NRP-V7 specific T cells in the islets (Figure 6)
Figure 6 NRP-V7+ CD8+ T cell levels in islets. Islets were isolated at 8 weeks for each group and pooled (N=4), each sample was also gated on CD3+ B220- cells.

Liposomal αGalCer + insulin (SC) and liposomal αGalCer only (IP) show two of the lowest levels of NRP-V7+ CD8+ T cells of all groups and were also two of the treatment groups that showed the most protection from T1D while liposomal αGalCer + insulin (IV), which was essentially identical in its protection compared to no treatment had the highest level of NRP-V7+ CD8+ T cells. However, the no treatment control group paradoxically showed the lowest levels of NRP-V7+ CD8+ T cells. When taken in context with the NRP-V7+ CD8+ T cell data in the blood as previously mentioned, there does seem to be a disconnect between NRP-V7 levels between the two organs. Outside of liposomal αGalCer + insulin (SC), which had comparatively low levels of NRP-V7+ CD8+ T cells in both the islets and the blood, both liposomal αGalCer + insulin (IP and IV) had relatively low levels NRP-V7+ CD8+ T cells in the blood but relatively high levels in the islets, which may explain why these routes of injections were inferior to subcutaneous injections.

3.5 Liposomal αGalcer + Insulin Has No Effect on Insulitis

Initial presumptions on the mechanism of action for liposomal αGalCer + insulin was that diabetogenic CD8+ T cells would not have an opportunity to destroy β
cells because activated Treg and/or NKT cells would inhibit their expansion with a suppressive cytokine milieu, render diabetogenic cells anergic or even be killed off via apoptosis. To test this theory, female NOD mice were given liposomal αGalCer + insulin treatment or controls as previously described and were sacrificed when they became diabetic or at week 30. Surprisingly, there was no correlation between disease outcome and insulitis score, with all treatment groups exhibiting roughly identical (and not statistically significant) insulitis scores (Figure 7).
Perhaps most striking was that liposomal αGalCer + insulin (SC) treated mice had the highest levels of insulitis of any group but conferred the most protection from T1D. There does appear to be a slight trend between disease outcome and total number of islets, with treatment groups with better survival curves having more islets; no treatment and liposomal αGalCer + insulin (IV) had the lowest number of islets counted, αGalCer + insulin (IP) and liposomal insulin control had moderate numbers of counted islets while the two treatment groups that fared the best, liposomal αGalCer + insulin (SC) and liposomal αGalCer control had the greatest number of counted islets. It is likely that the variance in numbers can be in part attributed to the fact that while fully infiltrated islets are easy to located as well as the difficulty of counting destroyed islets as the islet morphology is no longer present along with the fact that diabetogenic T cells migrate out of the islet once all the β cells have been destroyed. Insulitis scores were separated between mice that did become diabetic and mice that did not and no statistical difference was found (data not shown).

**Figure 7** Infiltration of islets after treatment with liposomal αGalCer + insulin

- a Representative scoring of insulitis ranging from 0 (no infiltration) to 4 (complete infiltration)
- b Total insulitis score of islets with total number of islets counted (N=8)
4 Immune Modulation by αGalcer + Insulin

4.1 Liposomal αGalcer + Insulin Expands Conventional Dendritic Cells In Spleen

Dendritic cells are the primary APC involved in CD1d mediated activation of iNKT cells and new studies have also shown that tolerogenic dendritic cells also may play a more direct role in preventing T cell mediated β cell destruction (Beaudoin et al. 2014). In order to determine if protection via liposomal αGalCer + insulin involved dendritic cells, planned sacrifices were scheduled at week 8 and week 16 due to their importance in the timing of disease pathogenesis; week 8 is roughly the start of major insulitis (Anderson & Bluestone 2005) while insulitis has typically completed destroyed the majority of β cells and clinical symptoms of T1D such as hyperglycemia become apparent by week 16. Conventional dendritic cells labeled with pan-DC marker CD11c while plasmacytoid dendritic cells labeled with both CD11c and mPDCA-1 (CD317) showed expansion compared to no treatment at week 8 with any treatment involving αGalCer, outside of pDCs in αGalCer + insulin (IV) mice which was only trending.

Furthermore, while dendritic cell populations were expanded by week 8 for cDC numbers returned back to no treatment baseline by week 16 (Figure 8). Interestingly, insulin appears to have no effect on expansion of either dendritic cell subset; insulin only controls did not see any at either time point while all treatment groups that included αGalCer did. Furthermore, while cDC numbers were more statistically striking a similar pattern could be observed with pDCs,
again while not statistically significant, treatment groups including αGalCer do have slightly higher pDC numbers.

**Figure 8** Expansion of dendritic cells in the spleen after liposomal αGalCer + insulin therapy. Female NOD mice were given injections of liposomal αGalCer + insulin or controls intravenously, intraperitoneally or subcutaneously from week 4 to week 9 twice weekly and sacrificed at week 8 and week 16. Statistical
significance was calculated by One-Way ANOVA test followed by Tukey’s test (*
p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001) a Percentage of CD3+CD11c+
dendritic cells (N=8) (ANOVA p>0.0001) b Percentage of CD3+CD11c^{int}CD317+
plasmacytoid dendritic cells. (ANOVA p=0.0002)

4.2 Liposomal αGalcer + Insulin Decreases Splenic iNKT but not in Treg

Cells
iNKT cells showed a decrease in subcutaneous injections of liposomal αGalCer + insulin at week 8 and a marked decrease in every treatment group at week 16. iNKT cells are known to down-regulate their TCRs rapidly after stimulation of αGalCer, which makes detecting NKT cells in as little as 2 hours after stimulation difficult,(Matsuda et al. 2000). It is possible that iNKT cells have already been activated, expanded and have been rendered invisible to CD1d tetramers by the week 16 timepoint.

Interestingly, Treg populations saw essentially no change in numbers at either week 8 or week 16. It’s possible that either the spleen is not an important location for the migration or expansion of Tregs or that the timing of the experiment does not accurately reflect Treg activity with αGalCer + insulin therapy. Expanded Tregs also are very short lived, only circulating about 1 to 2 weeks (Webster et al. 2009), and in humans also have difficulty maintaining steady FOXP3 expression after repetitive stimulation (Hoffmann et al. 2009).
**Figure 9** Decreased population of iNKT in the spleen after liposomal αGalCer + insulin therapy. Female NOD mice were given injections of liposomal αGalCer + insulin or controls intravenously, intraperitoneally or subcutaneously from week 4 to week 9 twice weekly and sacrificed at week 8 and week 16. Statistical significance was calculated by One-Way ANOVA test followed by Tukey’s test (*p*<0.05, **p*<0.005, ***p*<0.0005, ****p*<0.0001) **a** Percentage of CD3+ B220− CD1d+ tetramer iNKT cells (Week 8 ANOVA p<0.0001, Week 16 ANOVA p<0.0001) **b** Percentage of CD4+ FOXP3+ Treg cells. (Week 8 ANOVA p<0.0001, Week 16 ANOVA p=.0243)
4.3 Single Injection of Liposomal αGalcer Expands APCs

Based on the previous experiments, αGalcer but not insulin could expand dendritic cells and therefore further experiments were performed to determine if liposomal αGalcer was expanding other APCs and if there were any specific organs that these cells were residing in. Furthermore, since the inclusion of αGalcer into a liposome can alter how these cells recognize and phagocytose antigens, both aqueous and liposomal αGalcer were tested (Figure 10).
Figure 10 Expansion of APCs after single injection of liposomal αGalCer. A single subcutaneous injection of αGalCer in aqueous or liposomal form was given to female NOD mice (N=5) and then sacrificed after 24 or 72 hours. Statistical significance was calculated by One-Way ANOVA test followed by
Tukey’s test (* p<0.05, **p<0.005, ***p<0.0005) a Percent of MHCII⁺CD11c⁺ dendritic cells in the blood (ANOVA p=0.0014) b Percent of MHCII⁺CD11c^{int}⁺CD317⁺ dendritic cells in the spleen (ANOVA p=0.0028) c Percent of MHCII⁺CD11b⁺F4/80⁺ macrophages in the blood (ANOVA p=0.05) d Percent of MHCII⁺CD11b⁺F4/80⁺ macrophages in the spleen. (ANOVA p=0.0005)

Both aqueous and liposomal αGalCer did expand APCs, however, different subsets of APCs expanded in different organs. cDCs were found to only expand in the blood while pDCs only expanded in the spleen. Macrophages however expanded in both the blood and spleen. No expansion was seen in the pancreatic lymph nodes while CD3⁺CD19⁻ T and CD3⁺CD19⁺ B cells also saw no expansion in any organ. Expansion of all APCs was also very short lived, with maximal presentation always occurring at 24 hours and trending back towards baseline by 72 hours. The rate of expansion is essentially identical when comparing aqueous and liposomal αGalCer with the exception of macrophages in the spleen, where liposomal αGalCer has significantly more expansion compared to aqueous αGalCer at 24 hours.

4.4 αGalCer Presentation on APCs in Parametrial Fat

Adipose tissue was once considered immunologically inert tissue but multiple studies have now shown that adipose tissue plays an active role in the immune system and that cross-talk between adipocytes and immune cells can lead to
altered immune regulation (Fantuzzi 2005). Specifically, cell isolated adipose
tissue results in a fraction called the stromal vascular fraction, is know to contain
mesenchymal stem cells, endothelial precursor cells and anti-inflammatory M2
macrophages (Riordan et al. 2009). Furthermore, adipose tissue is known to
harbor unique subsets of Tregs (Feuerer et al. 2009) and iNKT cells also have
been found to be highly enriched in human and murine adipose tissue and these
fat-derived iNKT cells are more Th2 biased and produce significantly less IFN-γ,
more IL-4 and IL-10 compared to iNKT cells in the spleen or liver (Lynch et al.
2012). Since liposomal αGalCer + insulin injected (SC) was the only treatment
group to protect mice from T1D, parametrial fat, one of the major adipose depots
that is attached to the uterus and ovaries of female mice was collected since it is
also the adipose tissue closest to the tail base injection site for subcutaneous
injections and could be an important site of αGalCer presentation.

8-week-old female NOD mice were injected subcutaneously with a single dose of
aqueous αGalCer, liposomal αGalCer or empty liposome and sacrificed 24 or 72
hours after injection. αGalCer presentation was determined using a
αGalCer:CD1d complex antibody that binds only to CD1d molecules when
αGalCer is present in its lipid binding groove. Mutant murine line RMA-S and a
transgenically modified RMA-S which expresses high levels of murine CD1d
(RMA-S.mCD1d) were used as controls.
Figure 11 αGalCer:CD1d presentation on APCs in the stromal vascular fraction of parametrial adipose tissue. A single subcutaneous injection of αGalCer in aqueous or liposomal form was given to female NOD mice and then sacrificed.
after 24 or 72 hours. a Percent of MHCII⁺CD11c⁺ dendritic cells presenting αGalCer:CD1d complex (N=5, ANOVA p=0.0263) b Percent of MHCII⁺CD11b⁺F4/80⁺ macrophages presenting αGalCer:CD1d complex (N=5, ANOVA p=0.0105) c αGalCer:CD1d complex antibody staining with mouse T cell tumor cell line RMA-S and RMA-S transfected with murine CD1d (RMA-S.mCD1d) stimulated with aqueous αGalCer (N=5)

Aqueous αGalCer shows presentation on cDCs and macrophages in the parametrial fat but not the spleen, pancreatic lymph nodes or the blood, suggesting that αGalCer is not trafficking to other organs nor are any cells that can present αGalCer migrating to other organs. Presentation was also only statistically significant with aqueous αGalCer and not liposomal αGalCer, suggesting that liposomal αGalCer decreases the presentation capacity of APCs. Furthermore, presentation was only statistically significant at 24 hours and not 72 hours suggesting that presentation is very quick and diminishes back to base line by 72 hours. Furthermore, no presentation was seen on B cells or pDCs, which is surprising considering that pDCs expanded in both the longitudinal experiment (Figure 8) as well as with single injections (Figure 10). Since no expansion of any cell type was seen in the stromal vascular fraction, this would indicate that a single injection of αGalCer does not directly expand APCs. This would seem to suggest that two separate mechanism are occurring, αGalCer presentation in the SVF and APC expansion in the blood and spleen suggesting that DCs not
involved in αGalCer presentation may be indirectly expanded, possibly by activated iNKT cells (Beaudoin et al. 2014). Moreover, aqueous αGalCer appears to have stronger presentation compared to liposomal αGalCer however liposomal αGalCer does appear to have longer presentation (Figure 11).

5 Discussion and Conclusion

5.1 Liposomal αGalcer + Insulin Protects Mice From T1D

The overall goal of this project was to determine the efficacy of liposomal αGalCer + insulin in protecting NOD mice from T1D. Liposomal αGalCer + insulin is an improvement in T1D protection over aqueous injections of αGalCer and insulin separately. Encapsulating αGalCer into a liposome not only allows it to maintain its potency in protecting NOD mice from developing T1D, reduces the number of injections needed and halves the effective dose of αGalcer per injection needed for protection (Figure 4) compared to previous publications (Hong et al. 2001). While the data suggests that insulin may not have as profound an effect on T1D protection as αGalCer, only the combination of insulin and αGalCer was successful in protecting NOD Mice from T1D and may play a role in mechanisms not fully understood at this time.

Route of injection also is paramount to the protective capacity of the drug. It is known that different routes of injection lead to different drug depot and drug trafficking profiles; Radiolabeling experiments on IV injections of liposomal αGalCer have discovered that it first goes through the lungs before the majority
ends up in the liver while its presumed that IP injections leaves most of the drug directly in the liver while SC injections leaves a drug reservoir in the parametrial fat, possibly though the lymphatic system (personal communication, Omar Duramad). Furthermore, IV injections allow for much quicker drug trafficking compared to SC and IP injections, which is one possible explanation of why protection did not develop in NOD mice injected IV.

Liposomal αGalCer + insulin also appears to not provide protection from T1D by eliminating NRP-V7+ CD8+ T cells. NRP-V7+ CD8+ T cells in the blood did show reduced numbers at week 8 for mice treated with liposomal αGalCer + insulin by any route of injection however this did not correlate to overall protection.

Previous studies have show a proportion of NRP-V7+ CD8+ T cells greater than 0.5% at any time point was predictive of disease onset. However, NRP-V7+ CD8+ T cells go through a cyclic pattern of clonal expansion and decline, which might be a response to a self-antigen followed by elimination/suppression through regulatory immune cells such as dendritic cells, NKTs and Tregs (Trudeau et al. 2003). Overall, NRP-V7+ CD8+ T cell percentages in the blood are often too variable and time-dependent to really be an accurate predictor of disease progression.

A more concrete analysis of diabetogenic cells is observing infiltrated NRP-V7+ CD8+ T cells directly in the islets, however islet isolations done at week 8 showed NRP-V7+ CD8+ T cells were present in all treatment groups and at roughly the same proportions. This data corroborates with the data obtained in the blood
that there is no correlation between NRP-V7⁺ CD8⁺ T cell percentages in either organ and T1D onset in the context of liposomal αGalCer + insulin.

Furthermore, histological analysis determined there was no statistical difference in insulitis scores between any of the different treatment groups, which again agrees with the previous findings that its unlikely that liposomal αGalCer + insulin is protecting NOD mice by either diminishing the NRP-V7⁺ CD8⁺ T cells or inhibiting these cells from migrating to the islets. However, while NRP-V7⁺ CD8⁺ T cells were found in the islets, mice were able to maintain euglycemia and their islets did not lose morphology, a typical characteristic of islet destruction. One possibility is that regulatory cells like Tregs were activated by insulin epitopes created by the breakdown of the liposomal αGalCer + insulin by APCs and then migrated to the islets and suppressed T cell mediated β cell destruction, thus leading to infiltrated islets but no disease onset.

Conventional dendritic cell expansion after repeated injections also does show some correlation to disease protection; liposomal αGalCer + insulin injected SC and IP showed the most significant expansion of cDCs and were the two treatment groups that showed the most protection (SC injections protected mice from T1D while IP injections delayed onset). On the other hand, IV injections showed a markedly lower expansion of cDCs compared either SC or IP injections while pDC expansion in IV injected mice showed no statistically significant difference from no treatment mice.

Repeated injections of liposomal αGalCer + insulin also lowered the percentage
of iNKT cells in the spleen both at week 8 and week 16. This is not surprising since iNKT cells are well known to down-regulate their TCRs upon activation thus rendering them invisible to conventional tetramer staining. This would indicate that αGalCer can still presented by APCs to activate iNKT cells even when it has been embedded into a liposome. Single injections of αGalCer also showed significant expansion of cDCs, pDCs and macrophages in the blood and spleen, while αGalCer:CD1d presentation was found on macrophages and cDCs in the stromal vascular fraction. Interestingly, there appears to be two subsets of APCs that have different roles in the protection of T1D; cDCs in the SVF were found to present αGalCer on their CD1d molecules but were not found to have expanded while in the blood cDCs did not have any αGalCer:CD1d expression. pDCs showed similar responses, while pDC expansion was detected in the spleen, but no pDCs were found to be presenting αGalCer. Only macrophages show αGalCer presentation in the stromal vascular fraction and expansion in the blood and spleen. Overall, this might constitute a feedback loop where αGalCer presenting APCs activate iNKT cells, which in turn can activate and expand different APCs, which may have a protective effect on T1D (Beaudoin et al. 2014).

Perhaps most interesting is that the only significant presentation of αGalCer on APCs was found with aqueous αGalCer and not liposomal αGalCer. It has been proposed that by altering the size of αGalCer can increase its loading time and produce a biased Th2 response (Yu et al. 2005), however liposomal αGalCer does not have this effect. Its possible that liposomal αGalCer actually increases
the half life of the drug by storing it in a liposome. Overall, the differences between liposomal and aqueous αGalCer were largely minimal, meaning that other factors like half-life, processing differential αGalCer processing or loading into the lipid groove of CD1d could be more important.

5.2 Summary

This project highlights some of the advantages and disadvantages of liposomal technology; while liposomal αGalCer + insulin does protect NOD mice from developing T1D at a lower titer and with a more medically realistic injection schedule than previous studies (Hong et al. 2001), it also shows that variables including route of injection and timing of injection can drastically alter the pharmacodynamics and pharmacokinetics of a therapy, which directly lead to changes in efficacy. Furthermore, incorporating liposomes into a therapy also can change the interplay between cell types involved in protection; some of these mechanisms involved are not fully understood at this time. Specifically, this project has shown that dendritic cells are likely more important to αGalCer mediated protection than initially thought. Future studies looking at different subsets of dendritic cells, specifically tolerogenic dendritic cells and their activation and expansion would be useful in fully elucidating the role of dendritic cells in αGalCer therapy.

One specific shortcoming of all immunoregulatory therapies for T1D is that it still doesn’t account for the loss of β cells and thus must be administered before the majority of β cell death has occurred. The possible solutions include pairing
immunoregulatory therapies like \( \alpha \text{GalCer} + \) insulin with better predictive analysis or by administrating \( \alpha \text{GalCer} \) at a time point where insulitis has started to occur but enough \( \beta \) cells are still alive to maintain euglycemia (around 8 weeks in mice).

Nevertheless, preventative therapies for T1D are still useful in understanding the pathology of the disease as well as furthering drug development that may eventually lead to a cure that can be administered after onset of T1D.

Finally, the translation of T1D in NOD mice to human patients is famously difficult, with multiple “cures” published but very few successful translations. \( \alpha \text{GalCer} \) in particular has shown through a large number of publications its potency and effect on iNKT activation and autoimmune diseases in general but at current there are no FDA approved \( \alpha \text{GalCer} \) based treatments nearly 20 years after its discovery and currently there are no FDA clinical trials outside of liposomal \( \alpha \text{GalCer} \) in progress.

Future experiments of interest include developing liposomal therapies that include specific epitopes like IGRP or InsB\(_{9-23} \) instead of whole insulin, which might improve the suppression of specific diabetogenic cells. Others include altering the injection schedule to mimic a more clinically relevant scenario; this project’s schedule involved therapy starting right as islet infiltration is occurring, which unfortunately doesn’t manifest any clinical symptoms. In addition, some at risk individuals ultimately never develop T1D, so either more predictive assays must be developed or more realistically, T1D therapies that are effective the
moment clinical symptoms arise need to be developed. Based on the number of injections, liposomal \( \alpha \text{GalCer} + \) insulin has shown it has an extended protective effect compared to aqueous \( \alpha \text{GalCer} \) which might make a single injection therapy possible, which is considered the greatest goal of drug development. Radiolabelling experiments involving different routes of injections have been proposed that can determine exactly which organs are operating as drug depots and the relative timing of drug trafficking; with this information, the liposome formulation can be modified to either accentuate or diminish the effect of specific organs and cell types.

In conclusion, liposomal technology offers a novel and potential cost effective alternative to new drug development. It is becoming increasingly difficult to not only develop but fund new drug therapy, in 2007 only 19 new drug therapies were approved by the FDA constituting about \$2 billion US dollars each of research and development to clear FDA trials (Paul et al. 2010). Often times many promising drugs show efficacy but do not pass FDA clinical trials could be modified with liposome technology in order to retain potency but eliminate unintended side effects or toxicity issues. By reducing the cost of drug development pharmaceutical companies can sell their products at lower prices while still drawing a profit which would in turn lower the cost of universal health care in Canada and medical costs around the world.


Boudaly, S. et al., 2002. Altered dendritic cells (DC) might be responsible for


Eberl, G. et al., 1999. Tissue-Specific Segregation of CD1d-Dependent and CD1d-Independent NK T Cells. *Journal Of Immunology*.


Krishnamurthy, B. et al., 2008. Autoimmunity to both proinsulin and IGRP is required for diabetes in nonobese diabetic 8.3 TCR transgenic mice. Journal Of Immunology, 180(7), pp.4458–4464.


Krishnamurthy, B. et al., 2006. Responses against islet antigens in NOD mice are prevented by tolerance to proinsulin but not IGRP. Journal Of Clinical Investigation, 116(12), pp.3258–3265.


Mohr, S.B. et al., 2008. The association between ultraviolet B irradiance, vitamin D status and incidence rates of type 1 diabetes in 51 regions worldwide.


Immunology, 8(12), pp.1353–1362.


