THE ROLE OF ENDOPLASMIC RETICULUM STRESS IN TYPE 1 DIABETES: IDENTIFICATION OF GLUCOSE REGULATED PROTEIN 78 AS THE AUTOANTIGEN FOR BDC-2.5 T CELL CLONE.

by

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THE ROLE OF ENDOPLASMIC RETICULUM STRESS IN TYPE 1 DIABETES: IDENTIFICATION OF GLUCOSE REGULATED PROTEIN 78 AS THE AUTOANTIGEN FOR BDC-2.5 T CELL CLONE.

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Environmental triggers, such as viral infection and environmental toxins, have been proposed to initiate the autoimmune disease of Type 1 Diabetes (T1D), however, the mechanism is unknown. The identification of novel autoantigens may provide insight to the mechanism of environmental triggers and pathogenesis of T1D. I identified the antigen recognized by the diabetogenic BDC-2.5 T cell clone using a novel in vivo reconstitution system, Restricted Immune System via Adoptive Transfer (RISAT). In RISAT, immunodeficient mice are adoptive transferred with a single T cell clone and an open repertoire of B cells. Reconstituted mice are immunized with an antigenic protein preparation. This system will drive an antibody response to the cognate antigen for the T and B cell through the co-stimulatory pathways involved in linked recognition. For the BDC-2.5 RISAT, non-obese diabetic (NOD).Rag-/- mice were adoptive transferred with the diabetogenic BDC-2.5 T cells and NOD B cells and then immunized with an antigenic beta cell membrane preparation (βmem) to drive an antibody response. The resulting antibodies recognized the endoplasmic reticulum (ER) stress associated protein glucose regulated protein 78 (GRP78) from βmem. To determine if ER stress plays a role in the antigenic response of the BDC-2.5 T cell clone, the non-antigenic NOD insulinoma cell line, NIT-1, were treated with thapsigargin, which induces ER stress. The treatment of NIT-1 with thapsigargin led to increased GRP78 synthesis, correlating with antigenic recognition by the BDC-2.5 T cell clone.

The antibodies from the BDC-2.5 TCR-Tg recognizes a subset of GRP78 which is modified with phosphoserine. The data presented in this thesis demonstrates a mechanistic link between ER stress and environmental triggers leading to the initiation of TID through the novel autoantigen, GRP78. Also the technique, RISAT, can be used to identify additional potential autoantigens of isolated T cell clones in both T1D and other autoimmune diseases.

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PREFACE

I am reminded of a story my Mom tells me about when I was a child. It was the first day of kindergarten. My Mom came back at the end of the day to pick me up. She sees me in tears. Her first thought was that I hated school. How was she going to convince me to come back tomorrow? In between sobbing, I explained that we painted today. I thought we were going to learn something.

To everyone at the Diabetes Institute, Rangos Research Center, INTBP, CMP, everyone from the Trucco's to the Shari's, thank you. I wouldn't have been able to accomplish everything if Darleen did not corner me for my signature on form, my mice cages weren't clean or my redos of my manuscript weren't emptied from the trashcan. Special thanks to my thesis committee, Dr. Wendy Mars, Dr. Tim Oury, Dr. Nick Giannoukakis, and Dr. Massimo Trucco, thank you for keeping me on track. Everyone that has passed through the Piganelli Laboratory, thank you, Dr. Hubert Tse, Martha Milton-Sklavos, Jen Profozich, and Gina Coudriet, thank you for your help with everything, scientific and non-scientific. Jon, I know you were always looking out for me and doing what was in my best interest, thank you.

To those who saw my potential and challenged me, thank you. You have shaped who I am and will be. Mr. Murray (Silver Lake Regional High School Biology Teacher), Dr. Jeffery

Bowen (undergraduate mentor at Bridgewater State College), my parents (Dr. Kenneth E Roberts aka Dad and Linda Roberts aka Mom), my sisters (Sarah and Stephanie), and my husband, Jason.

To friends and family who love me and have dealt with me through this roller coaster called graduate school, thank you. Tara, thank you for our lunches. Gina, thank you for being my Pittsburgh sister. For my husband's family, especially Linda, thank you for everything you have done for my family and me. I was able to go to work knowing that my children were in good hands.

To my reason for getting up when I would love to sleep in, my family, thank you. Jason, thank you for my most precious gifts, Abigail and Caleb.

1.0 INTRODUCTION

1.1 DIABETES & GLUCOSE METABOLISM

Diabetes is a metabolic disorder that affects how the body metabolizes glucose due to altered insulin production or sensitivity. Typically, food is broken down into glucose, a form of sugar in the blood. The hormone insulin is responsible for facilitating the transport of the glucose into cells, which use it for energy and growth. As the main site of hormone regulation of blood glucose, the pancreas, an endocrine organ, is comprised of several cell types that secrete specific hormones to regulate blood glucose levels (**Figure 1.1**).

There are three pancreatic cell types, which secrete hormones into the blood to regulate blood glucose. The alpha cells secrete glucagon during a state of low blood glucose, or hypoglycemia. Glucagon signals the liver to convert glycogen into glucose for release into the blood. The beta cells produce and secrete insulin. Elevated blood glucose levels, or hyperglycemia, activates secretion of insulin. Insulin signals liver and muscle cells to take up and store excess glucose in the form of glycogen. This also leads to fat cell up take of blood lipids that are converted into triglycerides. Lastly, the delta cells produce somatostatin, which is responsible for the release of both glucagon and insulin. In individuals with normal metabolism, after a meal, insulin is released to activate cells to utilize and store glucose. After several hours when blood glucose levels are starting to decrease, glucagon is secreted causing the release of

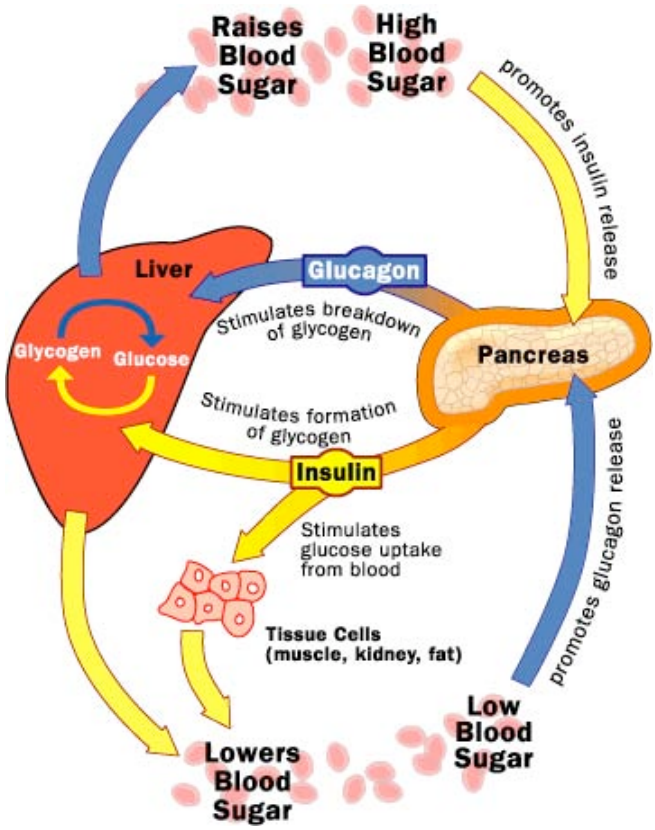

Figure 1.1 Pancreatic hormones regulate blood glucose. Regulation of blood glucose or sugar level is regulated by glucagon and insulin. Insulin acts on tissue cells to stimulate glucose uptake from blood lowering blood glucose. Low blood sugar promotes glucagon secretion. Glucagon stimulates the breakdown of glycogen to glucose in the liver. Glucose is released into the blood. High blood glucose promotes the release of insulin. [1]

[1] From www.howstuffworks.com/ diabetes1.htm Courtsey of HowStuffWorks.com

stored glucose. The importance of this ability to tightly regulate blood glucose levels has allowed humans to survive periods of feast and famine.

1.2 NON-AUTOIMMUNE DIABETES

There are 3 main types of Diabetes: Type 1 Diabetes (T1D), Type 2 Diabetes (T2D), and gestational diabetes. The primary difference between the three types of diabetes is how insulin is produced and metabolized. The most common form of diabetes is T2D with approximately 90 to 95 percent of the disease falling under this classification (1). Previously, T2D was only diagnosed in older individuals; however, more children and adolescents are being diagnosed with T2D. This shift in the onset of T2D correlates with an increasing population of overweight children. At onset, individuals with T2D produce insulin levels equal to non-diabetics, however they have lost the ability to utilize insulin efficiently and consequently suffer from insulin resistance.

The third type of diabetes, gestational diabetes, occurs in 3 to 8 percent of pregnant women in the United States during the last trimester. It develops due to insufficient insulin production and/or changes in hormones due to pregnancy. After the baby is born, gestational diabetes usually disappears. These individuals are at a higher risk of developing T2D.[2]

[2] http://www.diabetes.org/gestational-diabetes.jsp

1.3 TYPE 1 DIABETES

T1D, also known as Insulin-dependent diabetes mellitus (IDDM) or juvenile-onset-diabetes, accounts for 5 to 10 percent of diabetes in the United States (2). The disease is characterized by the autoimmune destruction of the islet-beta cells responsible for the production of insulin, typically, at a young age. The average age of onset is 20 years old with individuals as young as newborn to 30 years old (3). The destruction of the beta cell mass by the immune system leads to a lack of insulin production, unregulated glucose metabolism and hyperglycemia. As shown in **Figure 1.2**, diabetes is not clinically recognized until 80 to 85% of beta cell mass has been destroyed (2, 3), therefore, newly diagnosed patients are left with exogenous insulin injection or islet replacement therapy as their only available treatment option. As new cellular and genetic markers and therapies become available, the goal is to identify diabetes either before any damage is done to the beta cell mass or while the majority of the beta cell mass is intact.

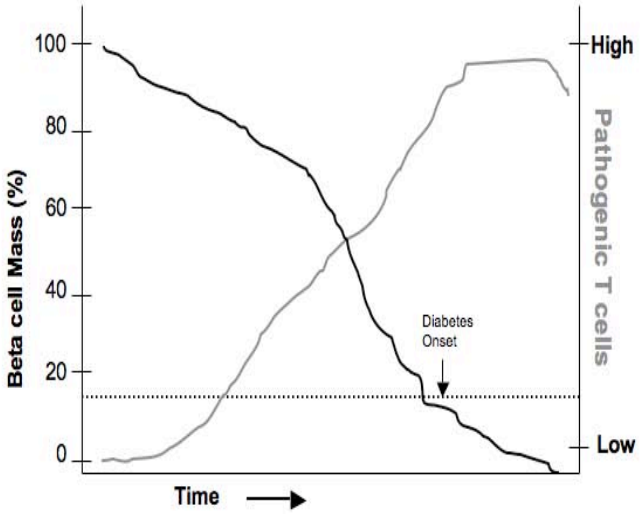

Figure 1.2 Beta cell loss corresponds with the level of autoreactivity in T1D. The progressive loss of beta cell mass (black line) correlates with an increased frequency of pathogenic $CD4^+$ and $CD8^+$ T cells (gray line). When approximately 85–90% of the beta cell mass has been destroyed, overt diabetes is manifested. Adapted from (4).

1.3.1 Insulin Replacement Therapy

Since first being isolated in 1920, exogenous insulin continues to be the primary treatment for T1D coupled with diet and exercise. There are two options patients have for treatment: multiple, daily subcutaneous injections or insulin pumps programmed to deliver insulin subcutaneously. Most recently, companies are beginning to test new devices that use a sensor, which is inserted under the skin to monitor glucose levels and signal the insulin pump when to deliver insulin. The sensor replaces the need for multiple finger pricks to read blood glucose levels. The sensor and pump work together in hope of creating an "artificial pancreas" that more closely mimics regulation of blood glucose.

Besides changes in how insulin is delivered, various forms of insulin have changed how individuals regulate their blood glucose with insulin replacement. The different forms of insulin

vary by their onset, peak time, and duration as outlined in **Table 1.I**. The combination of the various forms of insulin allow for better regulation of blood glucose levels.

Table 1.I. The types of insulin and their onset, peaktime, and duration.[3]

Type	Onset	Peaktime	Duration
Rapid-acting	5 min	1 hr	2 to 4 hrs
Regular/Short-acting	30 min	2 to 3 hrs	3 to 6 hrs
Intermediate-acting	2 to 4 hrs	4 to 12 hrs	12 to 18 hrs
Long-acting	6 to 10 hrs	No peak	20 to 24 hrs

1.3.2 Islet Replacement Therapy

Another more experimental treatment strategy is islet replacement. This potential therapy is achieved by either whole organ pancreas transplantation or isolated pancreatic islet transplantation. Islet transplantation has shown some success, 10% of patients at a 5-year follow-up are able to be insulin free (5). However, in order to avoid rejection, patients are put on an immunosuppressive regiment. Suppression of the immune system can lead to undesirable complications such as gastrointestinal problems, bacterial and viral infections, and cancer (6). Another challenge of transplant therapy is a shortage of islets available for transplantation. Of the 6,000-donor pancreata, less than half are suitable candidates for whole organ pancreas transplantation (6). Furthermore, the remaining pancreata are subjected to experimental

[3] Adapted form the American Diabetes Association website. www.diabetes.org

assessment to determine whether they can be used for islet isolation and transplantation into diabetic patients. When islets are isolated from the pancreas, two to four pancreata are needed to isolate enough islets to regulate blood glucose (6). Even if all donated pancreata were used for either whole organ or isolated islet transplantation, there would still be a shortage. The shortage of viable human organs has lead to emerging research on xenotransplantation, using non-human animals as organ donors. Some success has been shown with the use of pig islets for the xenotransplant (7).

1.4 GENETICS OF TYPE 1 DIABETES

It is clear that T1D is a polygenetic multifactorial disease. Although, the presence of T1D susceptible genes does not equate to 100% penetrance, the importance of genetic factors is unmistakable.

1.4.1 HLA Susceptibility

There are a number of genes that have been implicated in the development of T1D. One of the most studied markers is the human leukocytes antigen (HLA) or the human major histocompatibility complex (MHC) that is encoded on chromosome 6. This marker is an attractive target because it has been determined that it is the major locus for type 1 diabetes susceptibility providing up to 40-50% of the inheritable diabetes risk (1, 2). HLA-A, -B, and –C encode for the MHC Class I molecules that are expressed on most cell types and are critical for the presentation of endogenously derived antigens as a result of infection by intracellular

pathogens. They recognize and present antigens from sources the immune system sees as foreign, i.e. bacteria, viruses, parasites, self-proteins in autoimmunity and donor proteins in transplantation. HLA-DR, -DP, and –DQ encode for the MHC Class II molecules expressed on antigen presenting cells (APC). Antigens presented via Class II are recognized by $CD4^+$ T lymphocytes with Class I recognized by $CD8^+$ T lymphocytes.

T1D is an inheritable autoimmune disease with association between certain HLA loci leads to an increased susceptibility and/or protection against development of disease. Certain HLA loci such as DQB1*0602 alleles have demonstrated protection against T1D with 20% of the general population expressing this haplotype. Only 1% of type I diabetics express DQB1*0602 (8). While HLA-DR3 or DR4 are expressed by 45% of the general population, 94% of T1D patients express these HLA (3). It is interesting to note that the association of HLA with disease development decreases in strength with increasing age of onset (9).

1.4.2 Non-MHC Genes

Besides HLA, the genetic importance of insulin and other non-MHC genes has been researched. Polymorphisms in the IDDM2 locus are associated with risk of T1D. IDDM2 codes for variable nucleotide tandem repeat (VNTR) 5 of the proinsulin gene or INS-VNTR5. There are three classes of INS-VNTR5. Class I contains 26-63 repeats up to 570 bases in length. Class III contains 140-200 repeats up to 2,200 bases in length with the length of Class II falling between the lengths of Class I and Class III. Class III, or long-form of INS-VNTR5, is protective against development of T1D as it increases the amount of insulin messenger RNA expressed in the thymus, causing enhanced antigen-driven negative selection of insulin-specific autoreactive T cells. Class I INS-VNTR5 increases T1D susceptibility as it decreases the amount of insulin

expressed in the thymus (2). Additional genes have been investigated for their genetic role in the development of T1D, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), interferon induced with helicase C domain 1 (IFIH1), inositol 1,4,5-triphosphate receptor 3 (ITPR3), IL-2 receptor, protein tyrosine phosphatase nonreceptor type 22 (PTPN22) (1, 2). The 1858T allele of PTPN22, an inhibitor of T cell activation, leads to increased susceptibility for T1D. This same polymorphism also increases the risk for other autoimmune diseases such as rheumatoid arthritis and lupus erythematosus (10). CTLA-4 plays a vital role in the immune system as a negative regulator of T cell activation coded on chromosome 2 at locus IDDM12. The A6230G single nucleotide polymorphism in the 3' flanking region of CTLA4 has shown to have a strong correlation with T1D (11).

1.5 ENIVRONMENTAL FACTORS

It is apparent from identical twin studies that genetic risk is not enough to develop T1D. In less than 50% of cases, both identical twins develop T1D, indicating that environmental factors must play a critical role in penetrance of T1D (12, 13). Some potential triggering events that have been proposed are drug or chemical exposure, viral infection, and certain food exposure.

1.5.1 Viral Infections

There is increasing evidence that infection with enterovirus, cytomegalovirus, coxsackie virus, parvovirus B19, and rotavirus in genetically susceptible individuals may act as a triggering event. The process of such infections in initiating an autoimmune response is not fully

understood. There are several popular theories on how these infections lead to diabetes onset. Beta cells infected with viruses, due to a pancreatic tropism or random infections, are targeted by the immune system to clear the viral load from the host, thus killing the beta cells (14, 15). An infection may "accidentally" cause destruction of the beta cells, also known as molecular mimicry. Molecular mimicry occurs when the sequence of viral proteins and native proteins have high homology. The viral protein from coxsackie virus B4, 2C (CVB4-2C), shares homologous regions with glutamic acid decarboxylase (GAD65). Anti-GAD65 antibody from diabetic patients cross-reacting with CVB4-2C can lead to molecular mimicry, as CVB4-2C specific lymphocytes are not able to distinguish the two proteins from one another and thus, mount an immune response to the self-protein (16, 17). Another theory speculates that the beta cells are killed in a bystander mechanism. Viral-specific T cells are activated and release cytokines such as interferon-gamma (IFN-γ), the hallmark T helper (Th) cell cytokine. The production of IFN-γ could shift the balance between Th1 and Th2 cell lineages, pushing the generation of a previously non-activated diabetogenic Th1 cell to emerge. Additionally, antigen expression on the surface of the beta cell is enhanced by IFN-γ. Coupled with genetic predisposition, the autoreactive Th1 cell is able to interact with its cognate antigen and becomes activated leading to beta cell destruction (18, 19).

1.5.2 Noninfectious Environmental Triggers

Numerous studies have explored the role of noninfectious environmental triggers such as exposure to cow's milk, breast-feeding, soy or wheat products, environmental toxins and vitamin D intake with only anecdotal results. Individuals exposed to cow's milk (CM) in the first year of infancy are more likely to develop T1D. Conversely, individuals who are breast-fed for the first

year of infancy are less likely to become diabetic. There are several key differences between cow and human milk (13). Due to high levels of casein, the protein concentration is higher in CM. Beta-lactoglobulin (BLG), the main whey protein component in CM is not present in human milk. Bovine albumin differs in amino acid sequence from human. There are three amino acid differences between bovine and human insulin, which are present in both milks. These differences may lead to immune system activation in an individual with autoreactive T cells (13).

Evidence for many different environmental triggers has been proposed. It is very likely that just as there are many genetic components to T1D, there is not one single environmental trigger responsible for disease onset. More likely, there is a common cellular event between various environmental triggers that initiates T1D through the induction of autoantigens.

1.6 IMMUNOLOGY OF TYPE 1 DIABETES

As previously mentioned, T1D is an autoimmune disease. Autoimmune diseases are characterized by the immune system attacking self-tissue. There is a break in immunological tolerance to allow this to happen. Immunological tolerance is the process by which the immune system does not respond to an antigen and is important for preventing autoimmunity. Tolerance can be divided into three distinct forms: central tolerance, peripheral tolerance and acquired tolerance. As **Table 1.II** demonstrates there is a distinct difference between the maturation of T and B lymphocytes (20).

Table 1.II Mechanisms of Tolerance in B and T lymphocytes. Adapted from (20).

	B lymphocytes	**T lymphocytes**
Main site of tolerance induction	Bone marrow; periphery	Thymus; periphery
Tolerance sensitive stage of maturation	Immature (IgM^+ IgD^-) B lymphocyte	$CD4^+CD8^+$ thymocyte
Stimuli for Tolerance induction		
Central	High-avidity recognition of antigen in bone marrow	High-avidity recognition of antigen in thymus
Peripheral	Antigen recognition without T-cell help	Antigen presentation without co-stimulators; repeated stimulation by antigen
Main Mechanisms of Tolerance		
Central	Apoptosis (clonal deletion); receptor editing	Apoptosis (clonal deletion)
Peripheral	Anergy (blocked signal transduction); failure to enter lymphoid follicles	Anergy; apoptosis (activation-induced death); suppression

1.6.1 Central Tolerance

Central tolerance is the selection of immature adaptive immune cells, both T and B lymphocytes, which occurs during the differentiation of both subsets of immune cells (21, 22). After central tolerance selection, these T and B lymphocytes seed the periphery where they take up residence in peripheral lymph nodes and the spleen, as well as circulate throughout the body where peripheral mechanisms are able to regulate their expansion.

1.6.1.1 Principles of clonal deletion.

Clonal deletion is the main mechanism regulating central tolerance and is comprised of negative and positive selection (**Figure 1.3**). T lymphocytes undergo selection in the thymus. Self-peptides are presented on the MHC of cortical thymic epithelial cells. T cells with moderate affinity TCRs are signaled to differentiate and undergo positive selection; however, T cells with high affinity TCRs undergo negative selection, through an apoptotic mechanism. Apoptosis-promoting factor is upregulated, which leads to programmed cell death. Additionally, T cells with low affinity TCRs are also eliminated due to "death by neglect" as they are neither signaled to proliferate or apoptosis (21, 22).

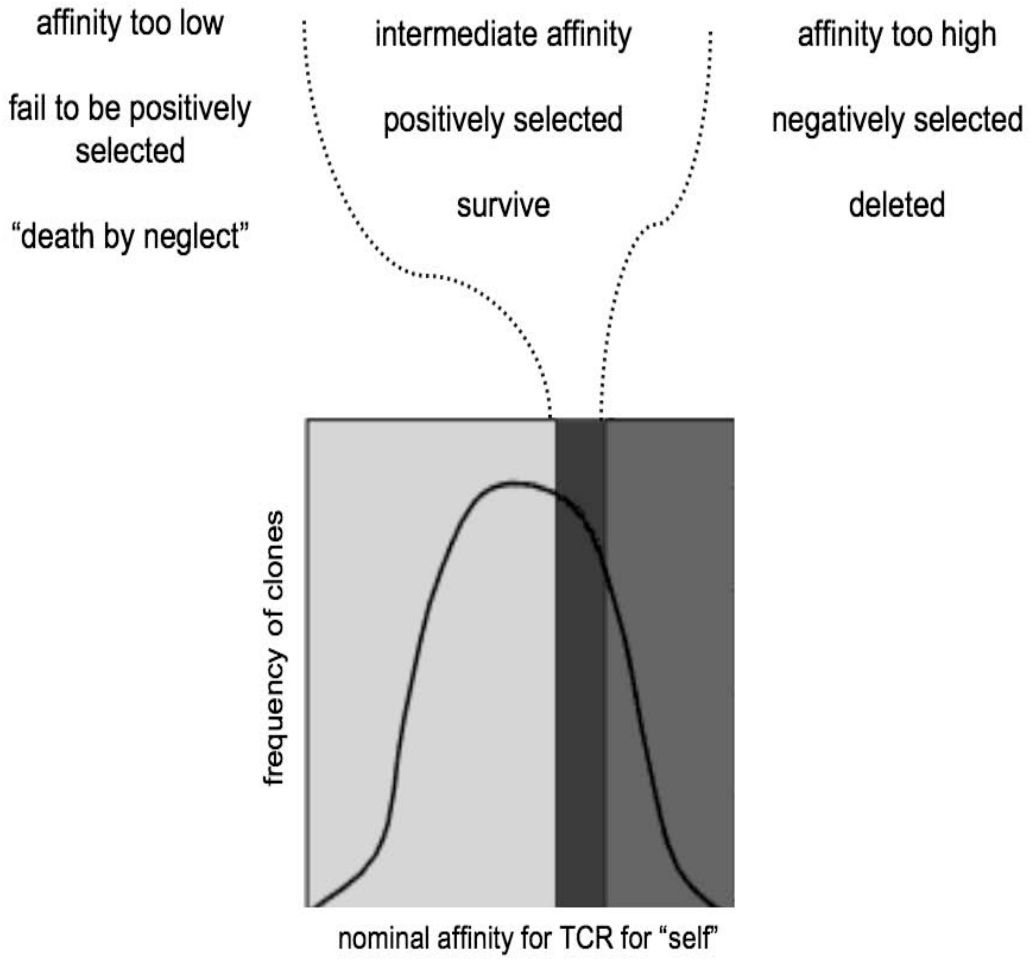

Figure 1.3 Clonal deletion of lymphocytes.[4]

[4] adapted from http://www-immuno.path.cam.ac.uk/~immuno/part1/lec08/lec8_97.html

1.6.2 Peripheral Tolerance

Not every protein in the body is expressed in the thymus or bone marrow, therefore, to further reduce autoreactive lymphocytes in circulation, lymphocytes are further selected against self-tissue once they have migrated into the periphery by peripheral tolerance mechanisms. There are at least four known mechanisms responsible for peripheral tolerance: anergy, ignorance, regulation and deletion.

1.6.2.1 Clonal Anergy and Adaptive Tolerance.

Anergy is the state of non-responsiveness by T and B cells to a specific antigen. In T cells, anergy is simply characterized by normal IL-2 receptor expression but insufficient IL-2 production (23). T cell anergy can be divided into two broad categories: clonal anergy and adaptive tolerance or *in vivo* anergy (24). Clonal anergy arises from incomplete T cell activation. A block in the Ras/MAP kinase pathway maintains this arrest state. T cells can be rescued from anergy by addition of IL-2 or anti-OX40 signaling. For $CD8^+$ T cells, clonal anergy does not inhibit the effector functions. In adaptive tolerance, naïve T cells are stimulated in an environment deficient in costimulation or high coinhibition occurring in either the thymus or the periphery. T cells proliferate and differentiate to varying degrees, however, in the presence of persistent antigen, T cells downregulate proliferation and differentiation. A block in tyrosine kinase activation, which inhibits calcium mobilization, results in anergy. Additionally, signaling through the IL-2 receptor is blocked. Adaptive tolerance reverses in the absence of antigen (25).

1.6.2.2 Regulation.

Regulatory T (Treg) cells achieve suppression of circulating lymphocytes. Treg can be natural occurring (nTreg) or adaptively induced. nTreg are $Foxp3^{pos}$ and selected for in the thymus. Although they recognize self-proteins, they do not lead to autoimmunity. Adaptive Treg cells exit the thymus as non-regulatory $Foxp3^{neg}$ T cells. In the periphery, these T cells under the right conditions are pushed to express Foxp3 and are able to suppress other immune cells. It is not fully understood what conditions are necessary to induce this switch. It appears that antigen-TCR stimulation and the presence of cytokines, such as TGFβ and IL-10 play a role in the switch from non-regulatory to regulatory T cells (26).

Various phenotypic markers have been identified. Initially, $CD4^+$ and $CD25^+$ were standard markers for Treg, however, if the immune system is already activated and/or in an inflammatory state, CD25 or IL-2 receptor is a poor marker as it is upregulated upon activation (26, 27). Low expression of IL-7 receptor α is used to identify human Treg (28). Glucocorticoid induced tumor necrosis factor (GITR), cytotoxic T lymphocyte associated receptor (CTLA)-4, lymphocyte activating gene 3 (LAG3), CD45RB and neuropilin are additional markers for Treg, however, they are also expressed on other T cell populations (26). The most specific Treg marker is the forkhead box transcription factor (Foxp3) (29-31). When traditional $CD4^+$ and $CD8^+$ are induced to express Foxp3, they are reprogrammed to become suppressors (32). Although, Treg secretes IL-10, direct contact, not secretion of cytokines, is required for suppression of other immune cells, primarily APC. CTLA-4 on the Treg surface triggers the production of indolamine 2,3-dioxygenase (33) that generates an immunosuppressive environment (34). Treg are present in T1D (35), however, it remains to be seen if there is a defect in T1D Treg that leads to immune system dysregulation.

1.6.2.3 Ignorance.

Ignorance occurs when a T or B lymphocyte fails to respond to its cognate antigen, even when the antigen is present in circulation. If the antigen is present at a low concentration, the receptor occupancy threshold that is necessary to trigger a response may not be met. Thus the lymphocyte does not sense the antigen. Another reason for ignorance is that the antigen is present in an immunologically privileged site, such as the eye, brain, testis and uterus. Cells in immunologically privileged sites express FasL on their surface, which upon binding signals cytotoxic T cells to apoptosis (36). Additionally, molecular mimicry can induce ignorance as the immune system sees foreign proteins as self due to sequence similarities.

1.6.3 Acquired Tolerance

Acquired tolerance is beyond the scope of this thesis, though for completeness, acquired tolerance is the immune system's tolerance for external antigens i.e. in transplantation. Acquired tolerance occurs through the mechanisms of ignorance, regulation, anergy, and apoptosis (37). Therapeutic treatments of transplant patients aim to induce tolerance to eliminate rejection of donor tissue.

1.7 B CELL DEVELOPMENT AND ANTIBODY MATURATION

Similar to T lymphocytes, B lymphocytes go through clonal deletion, however this is achieved in the bone marrow. Immature B cells express surface immunoglobulin M (IgM) as their antigen receptor or B cell receptor (BCR). If the IgM molecules are able to crosslink with their cognate

antigen during development in the bone marrow, the immature B cell undergoes receptor rearrangement, or receptor editing. If, after receptor editing, the immature B cell still recognizes self-proteins, it becomes apoptotic. This mechanism leads to immature B cells that do not recognize self-proteins in the periphery.

In the periphery, the immature B cells undergo further selection against self-proteins. Immature B cells that fail to react against self-proteins mature into mature B cells that express IgD in addition to IgM. The naïve B cells are able to bind foreign proteins or self-proteins on its B cell receptor (BCR). Upon recognition of its cognate antigen, the naïve B cell is activated and expresses CD40 on its surface (**Figure 1.4**). T helper cells, Th, are able to interact with the B cell through the CD40L and CD40 (38). This interaction is only achieved if the Th and B cell are able to bind their cognate antigen at the same time. The B cell will present the antigen on its MHC class II allowing the Th cells to bind the antigen on its T cell receptor (TCR). The Th cell secretes cytokines to stimulate proliferation of the B cell. The release of IFN-γ promotes IgG isotype switching (39). The B cell matures to a plasma cell that secretes IgG antibodies, which are able to diffuse into the blood and tissues (36, 40).

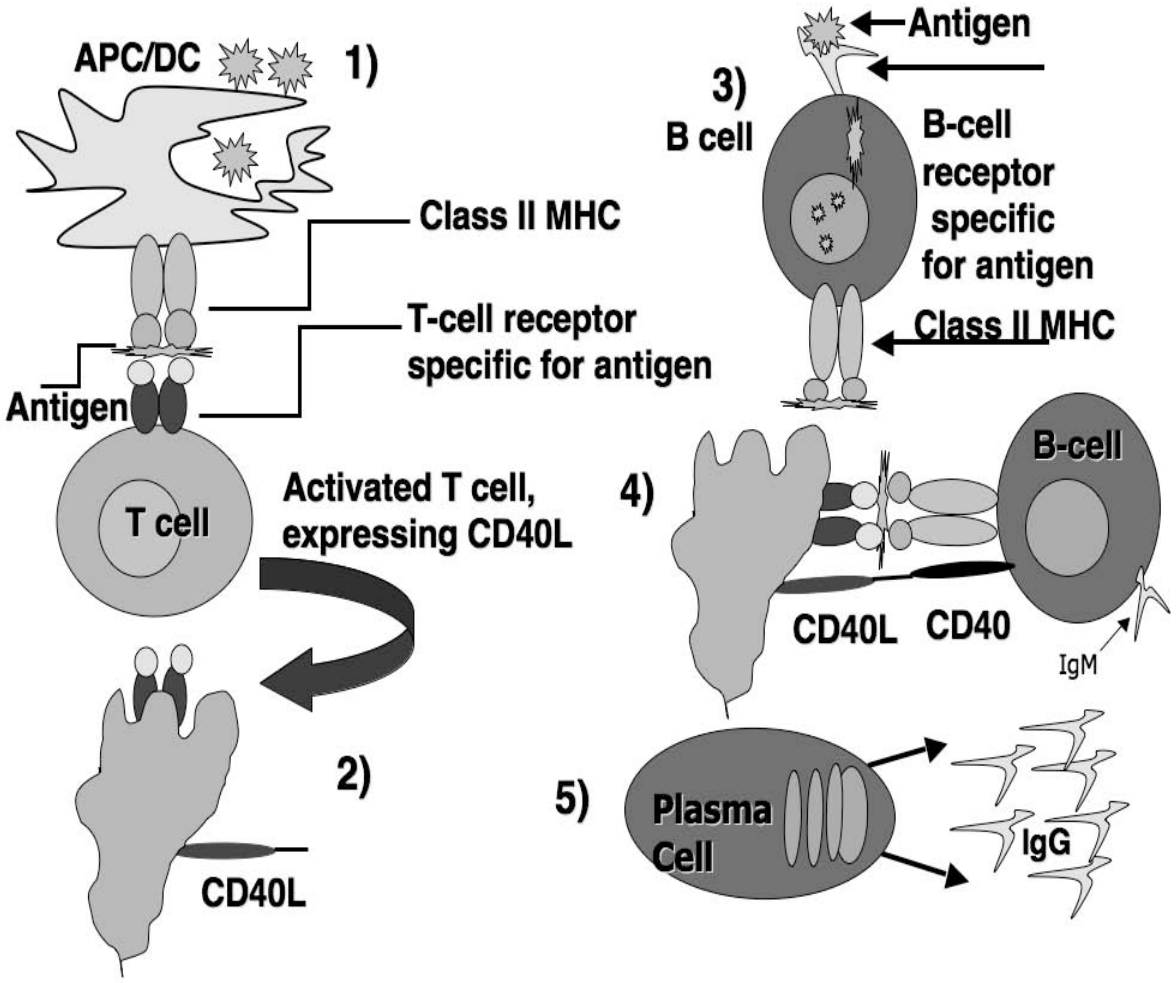

Figure 1.4 Interactions of T and B cell in antibody isotype switching.

1.8 AUTOIMMUNITY: BREAKDOWN OF THE IMMUNE SYSTEM

Autoimmunity is characterized by a break in both central and peripheral tolerance resulting in the recognition of self-tissue as foreign. The immune system is a dynamic system with the above-mentioned mechanisms occurring simultaneously. In autoimmunity, it is not the failure of a specific branch of tolerance that leads to the progression of this disease (41). In T1D, autoreactivity ensues as a result of the escape of self-reactive T cells that recognize beta cell antigens. Autoreactive B cells fail to be selected against in the bone marrow. Once in circulation the autoreactive lymphocytes evade peripheral tolerance detection. When B cells come across their cognate antigen, the lymphocytes are stimulated and proliferate. The B cells will produce autoantibodies that mark the beta cells for destruction. Though Treg are present in the circulation, they are unable to suppress the autoreactive lymphocytes from initiating the destruction of beta cells (26).

1.9 AUTOANTIBODIES & AUTOANTIGENS ASSOCIATED WITH T1D

Islet-cell cytoplasmic autoantibodies (ICA) were first discovered in T1D patients in 1974. Sera from type I diabetic patients revealed detectable islet proteins in frozen pancreas sections by indirect immunofluorescence. Two of the ICA antigens were identified as glutamic acid decarboxylase (GAD65) and insulinoma-associated protein 2 (IA-2). The ICA immunofluorescence test was commonly used as the diagnostic gold standard until recombinant proteins for GAD65 and IA-2 became available (3, 42). The advent of the highly sensitive and less cumbersome radioimmunoassay with recombinant GAD65 and IA-2 allows for a high-

throughput assay to determine whether a patient is autoantibody positive. This assay set a major milestone in the progression toward identification of patients at risk for progression to T1D prior to disease onset. Insulin was identified as an autoantigen later in the development of T1D because during the preparation of the frozen section slides for ICA immunofluorescence test, insulin, along with c-peptide, is leached from the cytoplasm of the sections. At present GAD65, IA-2 and insulin autoantibodies are standard markers for diagnosis of T1D. These antibodies are also used to assess an individual's risk of developing diabetes (**Figure 1.5**). GAD65 and IA-2 positive individuals demonstrate a 50% likelihood of developing T1D within 5 years (43). When all three autoantibodies are present, the risk of developing diabetes within 5 years increases to 70% (44).

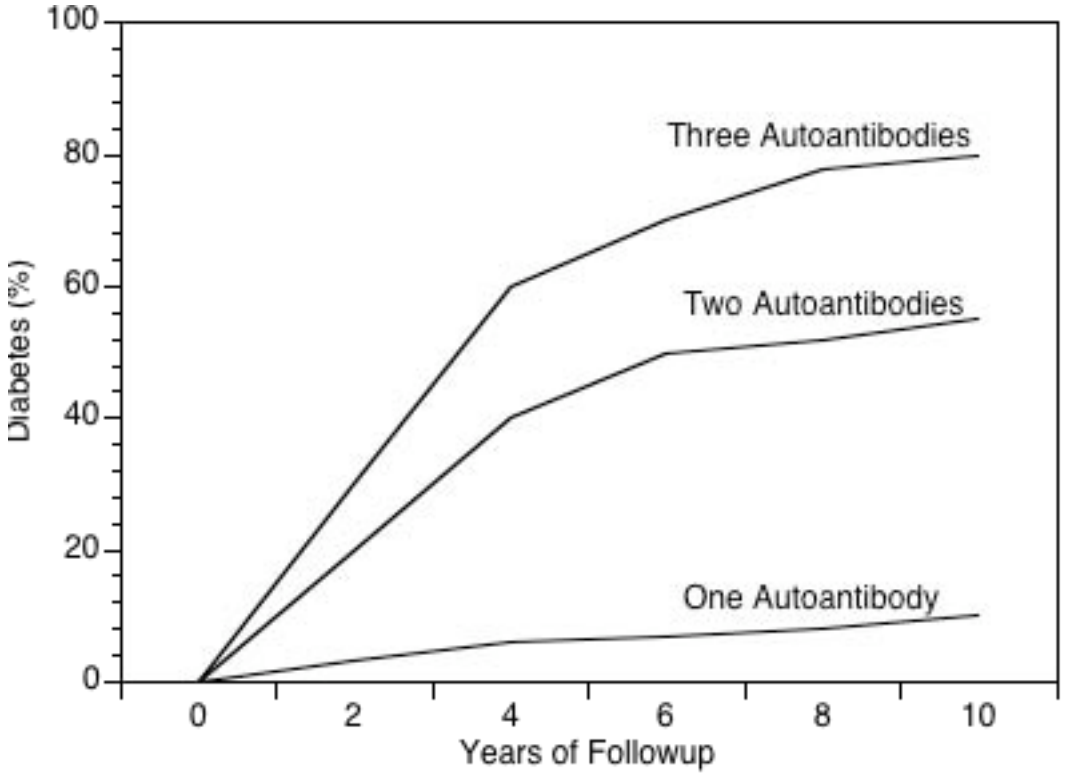

Figure 1.5 Diagrammatic representation of the effect of multiple autoantibodies (GAD65, IA-2, or insulin) on the risk of developing T1D. Percent diabetes represents an approximation from several longitudinal studies on first-degree relatives of T1D patients. Adapted from **(3).**

1.9.1 Glutamic Acid Decarboxylase (GAD65)

More specifically, GAD is expressed in two isoforms, GAD65 and GAD67. The molecular weight of GAD65 and GAD67 are 65 kDa and 67 kDa, respectively and the isoforms are 65% identical. GAD functions to convert glutamic acid to gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter. Both isoforms are equally expressed in neurons, however, GAD65 is predominately expressed in the beta cells. In beta cells, GAD is modified by lipids and anchored to the cytosolic face of synaptic-like microvesicles that store and secrete GABA. Autoantibodies present in the sera of type I diabetics recognize the middle (amino acids 245-449) and C-terminal (amino acids 450-585) regions of GAD65, which is 585 amino acids long. There is some cross-reactivity with the autoantibodies to GAD67 (3). GAD65 autoantibodies are present at the onset of diabetes and in at-risk first-degree relatives of type I diabetics (42). Thus, the presence of these autoantibodies provides limited insight to the pathogenesis of the disease.

1.9.2 Insulinoma-associated protein 2 (IA-2)

IA-2, also known as ICA-512, is a member of the receptor-type protein tyrosine phosphatase family. The predicted structure of IA-2 contains a luminal domain (amino acids 1-576), a single transmembrane domain (amino acids 577-601) and a cytoplasmic tail (amino acids 602-979), which includes a tyrosine phosphatase motif (42). Unlike other members of the receptor-type protein tyrosine phosphate family, IA-2 lacks a functional catalytic domain due to an Ala to Asp substitution at position 911 (3). In addition to the pancreatic alpha and beta cells, IA-2 is expressed in neuroendocrine cells and is localized to the secretory vesicles of both cell types. It

is speculated that IA-2 is involved in the trafficking and maturation of insulin secretory granules as IA-2 is found in insulin granules membranes (42).

1.9.3 Insulin

Insulin autoantibodies recognize "conformational" epitopes, which is an epitope that is only present when the protein is folded in a tertiary structure. This is also the case with GAD65 and IA-2 autoantibodies. The A and B chain of insulin is derived from preproinsulin (**Figure 1.6**). The leader peptide is removed from preproinsulin and two endoproteolytic cleavages excise the c-peptide region. The B chain is processed by carboxypeptidase E (CPE) to yield its final form. The A and B chain are held together covalently with two disulfide bonds. Autoantibodies, $CD4^+$ and $CD8^+$ T cells specific for insulin, all recognize epitopes on the B chain. Due to the formation of antibodies to exogenous insulin following insulin replacement therapy, analysis of the presence of insulin autoantibodies needs to be done prior to insulin replacement.

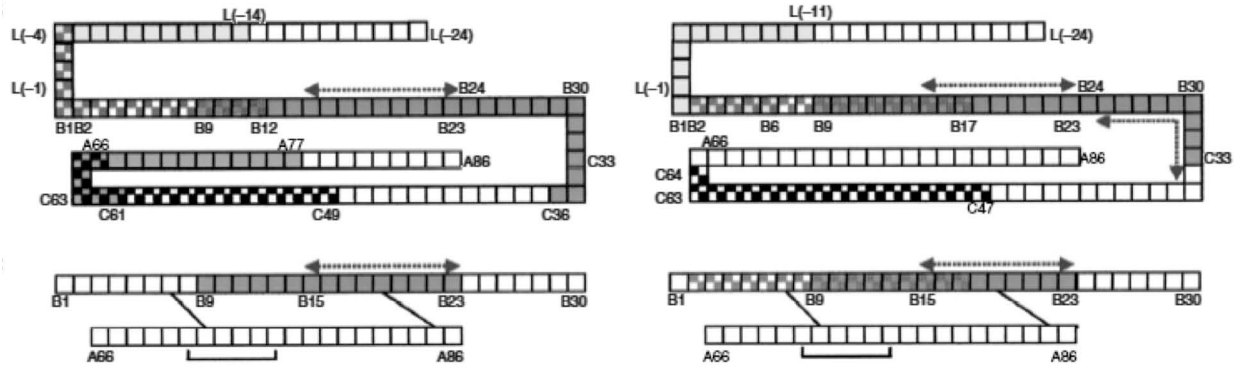

Figure 1.6 T-cell epitopes for insulin, the 'original' autoantigen. Human preproinsulin (top left) and murine preproinsulin 2 (top right). Epitopes for $CD4^+$ T cells are indicated by different shading patterns, and the beginning and ending residues for each epitope are labeled. Broken red arrows denote $CD8^+$ T-cell epitopes. As is true for certain other beta cell antigens, certain preproinsulin peptides are antigenic in both humans and NOD mice (including the $CD4^+$ T-cell epitope B9–23), and $CD8^+$ T-cell epitopes overlap those for $CD4^+$ T cells. Human insulin (bottom left) and murine insulin 2 (bottom right). Disulfide bonds are represented by solid black lines. Adapted from **(42).**

1.9.4 Islet Cell Autoantigen of 69 kDA (ICA69)

The autoantibodies of islet cell autoantigen of 69 kDA (ICA69) have the potential to yield tremendous insight regarding the pathogenesis of the T1D, as they are detectable in prediabetic individuals. ICA69 was first discovered from the sera of three prediabetic relatives of a T1D patient; all three later became diabetic (45). Screening of sera from subjects at risk for T1D were positive for ICA69 autoantibodies in 43% (10 of 23) of ICA^+ first-degree relatives, who later developed T1D. The potential of ICA69 being a marker indicative of development of T1D, was somewhat undermined by studies conducted in the non-obese diabetic (NOD) mouse, which demonstrated that $ICA69^{null}$ NOD mice developed diabetes with the same kinetics as wild-type NOD littermates (46). It, however, has yet to be determined if the role of ICA69 is the same in humans, but it remains a very hot topic in T1D research.

1.9.5 Other autoantigens

Numerous other autoantigens have been identified through autoantibodies and autoreactive $CD4^+$ and $CD8^+$ T cells. The target antigens are located in a variety of compartments with various functions in the beta cells as shown in **Figure 1.7**. Nine out of twenty-four antigens are associated with the insulin secretory granules. Heat shock protein 70 (HSP-70) and islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) are two autoantigens that are primarily located in the ER and thus are of special interest in this thesis.

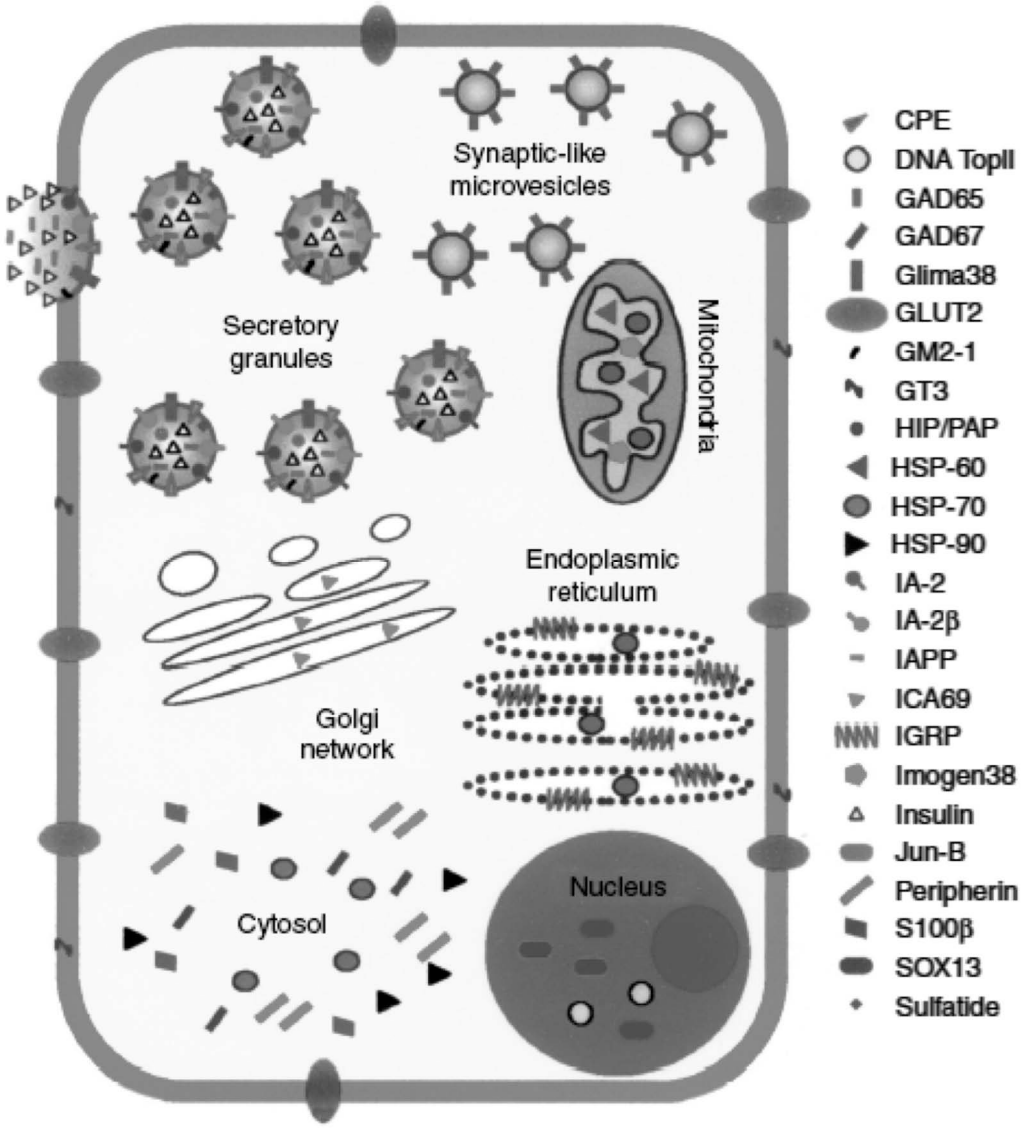

Figure 1.7 Subcellular localization of beta cell antigens. The proteins and lipids to which T1D patients and/or NOD mice have demonstrated immune responses are each denoted by a different symbol in this schematic representation of a beta cell. The position of each symbol within the beta cell indicates the cellular compartment in which that particular antigen is primarily localized. Although antigens are located throughout the beta cell, the highest number sof antigens (nine of 24) are associated with the secretory granules **(42).**

1.9.5.1 Heat shock protein 70 (HSP70).

HSP70 antibodies and autoreactive T cells have been detected in children (under the age of 18) with T1D. Two major epitopes, p27 (amino acids 391-410) and p35 (amino acids 511-530), have been identified for HSP70 (47). It is not known how HSP70 becomes antigenic, however, it has been documented that even control individuals have some detectable IgG specific-antibodies for HSP70 with 45% of type I diabetic children expressing significantly higher HSP70 IgG levels (47). Abulafia-Lapid et al proposes that this may be due to immunological homunculus (48), naturally occurring autoimmunity in healthy individuals (47). One could speculate that aberrant peripheral regulation of these naturally occurring autoimmune cells contributes to organ-specific autoimmunity of T1D.

1.9.5.2 Islet-specific Glucose-6-phosphatase catalytic subunit-Related Protein (IGRP).

IGRP specific $CD8^+$ T cells have been isolated from NOD islets at the earliest stages of detectable islet infiltration (49), supporting the notion that IGRP may be an early target in the pathogenesis of T1D. Additionally, the presence of $CD4^+$ and $CD8^+$ T cells and the $CD8^+$ T cell epitopes have been confirmed in humans and NOD mice (49-52). Most recently, a considerable effort has been put forth toward understanding the role of ER stress in the induction of increased IGRP expression (53).

1.10 TYPE 1 DIABETES MOUSE MODEL

1.10.1 Non-Obese Diabetic (NOD) Mouse Model

With the diagnosis of diabetes being made when approximately 80% of beta cell mass has been destroyed by the immune system, animal models have played a pivotal role in elucidating the pathogenesis of T1D. The non-obese diabetic (NOD) mouse is the most commonly used mouse model in T1D research (12, 42, 54, 55). The NOD mouse was developed unexpectedly through the breeding of a cataract-free CTS (Cataract Shionogi mouse) strain (12, 56). The utility of the NOD mouse model is born out of the similarities this strain has to human T1D, in that NOD and humans spontaneously develop diabetes, produce islet-specific antibodies, and generate T cells that recognize islet-beta cell associated antigens leading to beta cell destruction. Beta cell loss leads to hyperglycemia and clinically apparent diabetes. The presence of multiple susceptibility loci has been associated with the progression of T1D in the NOD mouse. For example Idd1 has mapped to the MHC II region. The NOD mouse has a distinct haplotype at the MHC II, which is characterized by the unique class II molecule I-A^{g7}. This class II molecule is unique in that the aspartic acid at position 57 in the ß-chain is substituted with a non-aspartic acid residue, which has been hypothesized to play a major role in the predisposition to T1D (12). Furthermore, it is known that NOD mouse's macrophages have a number of defects including processing and presentation (57, 58). Specifically, NOD macrophages are defective in engulfing and clearing apoptotic bodies that result from dead cells. It is proposed that poor clearance leads to the presentation of self-proteins to the autoreactive T and B cells. Approximately 80% of the female

and 20% of male mice in a NOD colony are diabetic by 6 months of age; however, the average age of onset is 12 weeks of age (12, 54). The sex difference in the diabetes incidence is unique to the NOD. It has been proposed that this is due to the effect of sex hormones. This is supported by studies in which castration increases the incidence of diabetes in male mice. For female mice, ovariectomy and administration of testosterone decreases the incidence of diabetes (59, 60). These studies demonstrate that increased estrogen levels lead to higher levels of T1D incidence and testosterone has a protective effect.

1.10.2 BDC-2.5 T Cell Clone

A number of cellular reagents from the NOD mouse have been isolated and utilized experimentally to elucidate the mechanism of T1D. For example, the generation of autoreactive T cells have been instrumental in increasing our understanding of the T cell mediated pathogenesis that occurs in the T1D autoimmune attack. One particular panel of T cell clones that have been studied extensively is the panel of autoreactive T cells outlined in **Table 1.III**. These autoreactive T cells induce diabetes when adoptively transferred into mouse strains such as the NOD, allowing studies to investigate early pathogenesis of T1D before disease onset. One diabetogenic T cell clone that has been extensively researched is the BDC-2.5 T cell clone, derived in 1989 (61). The BDC-2.5 T cell clone has a known T cell receptor (TCR), vβ4 vα1, and was originally derived from the spleen and lymph node of a newly diabetic NOD female. A beta granule membrane antigen is present in the context of the NOD MHC class II IA-^{g7} (62).

Table 1.III Islet-specific T cell clones. Adapted from (61).

			In vitro response to NOD APC and islet-cell antigen					
Clone	CD4/ CD8	TCR	No Ag	NOD IC	Non-NOD IC	Rat IC	βTC3	Islet graft damage
BDC-2.4	+/–	n.d.	+	+	+		+	–
BDC-2.5	+/–	Vβ4 Vα1	–	+	+	–	+	+
BDC-4.6	+/–	n.d.	–	+	+	–		+
BDC-4.12	+/–	Vβ19 Vα[4.12]	–	+	+			
BDC-5.2	+/–	Vβ6 Vα12	–	+	+	+		
BDC-5.10	+/–	Vβ4 Vα(n.d.)	–	+	+	–	+	+
BDC-6.3	+/–	Vβ4 Vα3.1	–	+	+	–	+	+
BDC-6.9	+/–	Vβ4 Vα13.1	–	+	–			+

Ag, antigen; IC, islet cells; n.d. not determined. + = positive response – no response from the T cell clone

Culturing the clone with islet cells and NOD APCs induces the production of IL-2, IFN-γ, and TNF-α, but not IL-4, indicative of a Th1 phenotype. When adoptively transferred into NOD or NOD.*scid* mice between 7 to 14 days of age, animals develop extensive insulitis and hyperglycemia. However, if the NOD recipient is over 3 weeks of age or an adult NOD.*scid*, a co-transfer of diabetic splenocytes needs to accompany the clone to successfully induce diabetes. Insulitis and hyperglycemia is a result of an autoimmune pancreatic infiltrate consisting of $CD4^+$ T cells, $CD8^+$ T cells, B cells, and macrophages.

1.10.3 BDC-2.5 TCR-Transgenic Mouse Model

To more invasively investigate the role BDC-2.5 plays in diabetes development, the BDC-2.5 T cell has been developed into a TCR-transgenic mouse. The BDC-2.5 TCR-Tg has been bred onto the NOD, NOD.*scid* and $B6^{g7}$ backgrounds (**Figure 1.8**). On the NOD background, only 10-15% of BDC-2.5/NOD animals spontaneously develop diabetes. By 3-5 weeks of age 100% of BDC-2.5 mice bred to the NOD.*scid* background develop diabetes (63). The BDC-2.5/$B6^{g7}$ begins to develop diabetes at 3 weeks of age, with the average onset at 6 weeks. The diabetes incidence at 10 weeks is approximately 60% in the BDC-2.5/$B6^{g7}$. BDC-2.5/$B6^{g7}$ animals that were free of diabetes at 10 weeks of age remain resistant (64).

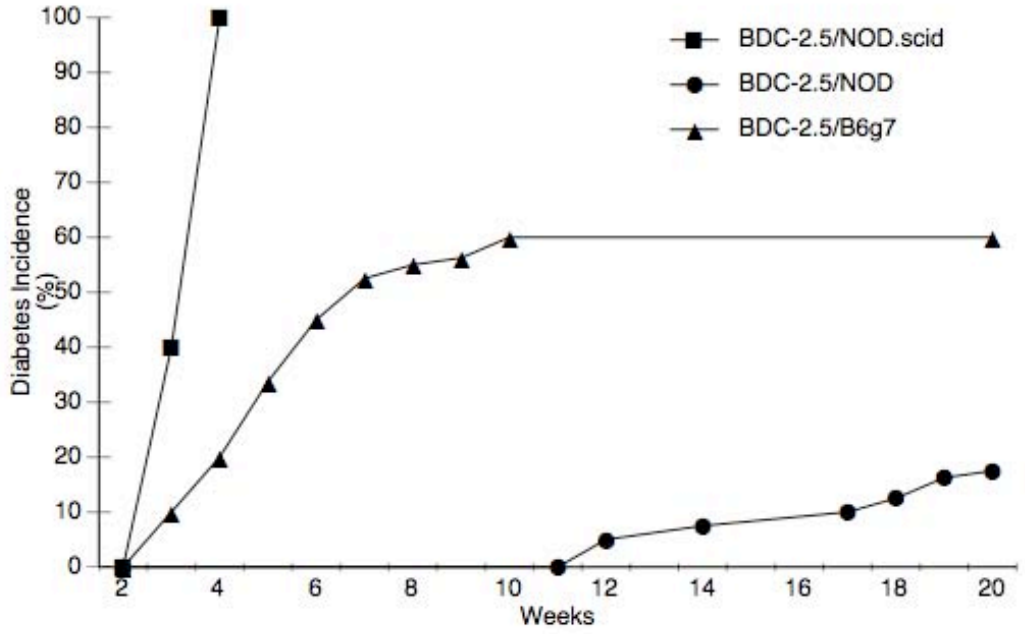

Figure 1.8 Diabetes Incidence for the BDC-2.5 TCR-Tg on NOD, NOD.*scid*, and B6^{g7} backgrounds. Adapted from (63-65).

1.11 PROTEIN TRAFFICKING

Protein trafficking is critical to the cellular well being due to the various compartments of the cell. The targeting of newly synthesized proteins to their correct compartment or organelle is achieved by sorting or signal sequences. In the absence of a specific signal sequence, protein is transported to the cytoplasm. Nuclear proteins are targeted by nuclear localization signal and are directly translated into to the nucleus through the nuclear pore. Resident endoplasmic reticulum (ER) proteins contain a "KDEL" sequence that retains it in the ER.

Briefly, the translation of mRNA in the cytoplasm occurs on free ribosomes. Signal sequences can either be translated in a continuous stretch of 15-60 amino acids or in a signal patch. Signal patches are comprised of several shorter stretches of amino acid that, when the protein is folded, signals the protein to be transported to the correct compartment (**Figure 1.9**). These signal sequences allow for signal recognition particles (SRP) to bind ribosomes and halt further translation. The SRP binds to docking proteina on the rough ER (RER) (65) and the signal sequence is inserted into the RER. With the protein synthesis resuming, the polypeptide chain is pulled through the membrane. The newly synthesized protein is now in the lumen of the ER. Some signal sequences are cleaved from the protein by signal peptidases. Signal patches generally are not removed from the protein. The ribosomes and SRP are released and recycled for further protein synthesis. In the ER, proteins undergo the posttranslational modifications of N-linked glycosylation and GPI-linkage. It is important to note that only proteins that are correctly folded are transported out of the ER. Misfolded proteins are bound by molecular chaperones and targeted for degradation in proteasomes. Further posttranslational modifications and sorting occurs in Golgi. Protein enters the Golgi on the cis face. As the protein is processed, it is transported through the Golgi to the trans face. Resident ER proteins are returned to the ER

in the vesicles. Secretory proteins are also packaged into vesicles for export to the cell surface (66).

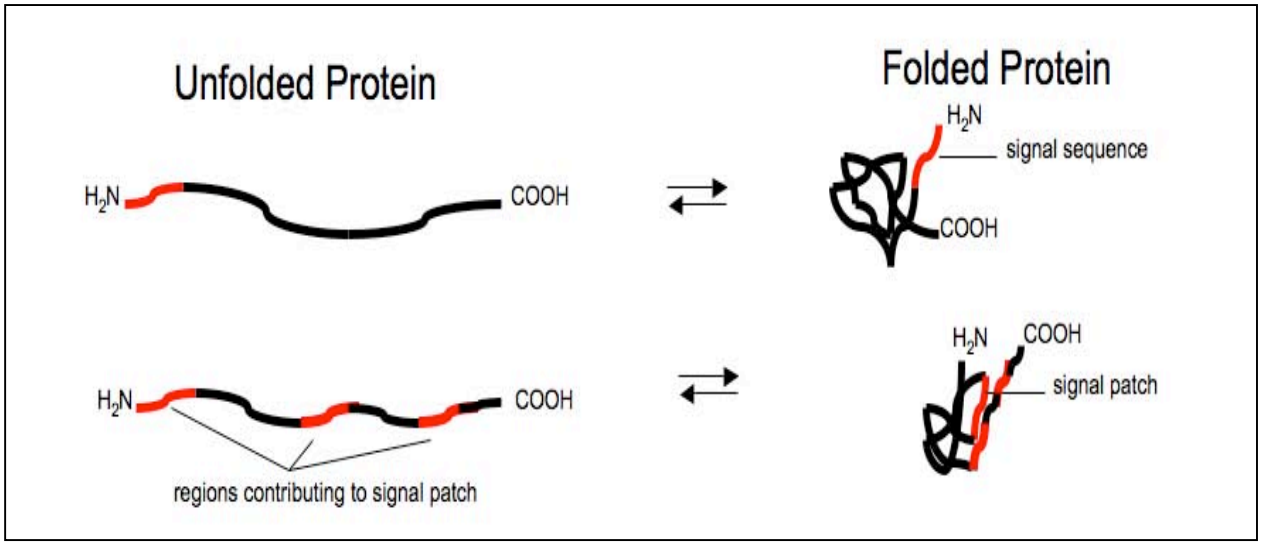

Figure 1.9 Signal Sequences. A) A signal sequence can be a continuous stretch of animo acids. B) Or a signal patch may be responsible for the targeting of a protein. Signal patches are made up of several regions that, when the protein is folded, signal for transport. Adapted from (66).

1.12 ENDOPLASMIC RETICULUM STRESS PATHWAY

The cellular organelle, ER, is an interconnected network of tubules, vesicles, and cisternae that connects the nucleus to the Golgi. As previously mentioned, the ER is the site of protein translation, protein folding, and posttranslational modifications. Secreted and membrane associated proteins pass through the ER on their way to the Golgi for further processing. The ER also acts as a storage location for calcium, glycogen, steroids, and other macromolecules (67). Importantly, the ER contains numerous molecular chaperones and catalysts to aid in the folding and posttranslational modifications of proteins (68).

Cells have developed signaling pathways and effector mechanisms to deal with the temporal and developmental variation in ER protein load. The ER stress pathway regulates this balancing act in four specific ways, 1) induction of ER chaperones through the unfolded protein response (UPR), 2) translational attenuation, 3) ER associated degradation (ERAD) (69), and 4) apoptosis (**Figure 1.10**) (70).

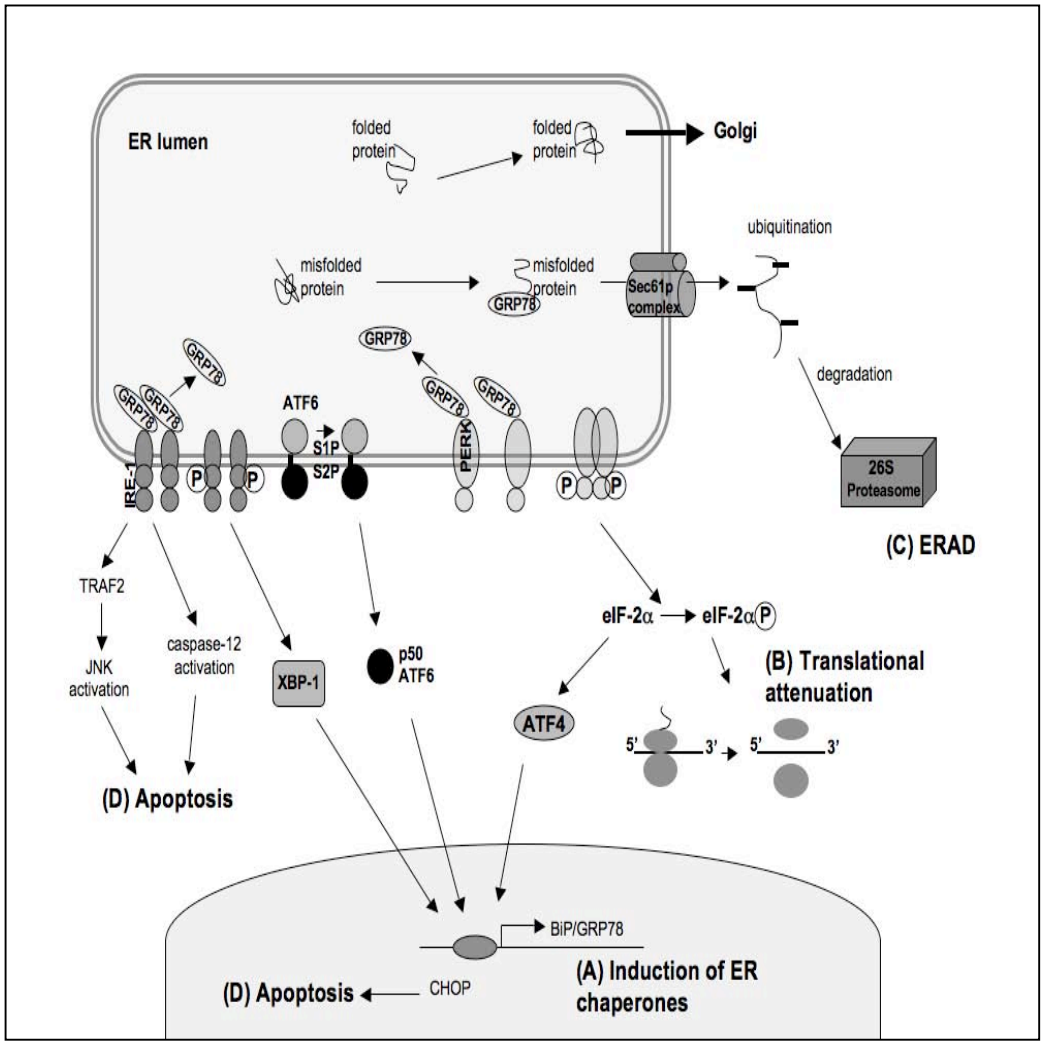

Figure 1.10 The ER stress pathway. In order to cope with the buildup of misfolded proteins, there are four cellular responses. A. Induction of ER chaperones and accessory proteins. B. Translational attenuation. C. Misfolded proteins are degraded through ERAD. D. Activation of apoptosis. Adapted from (70).

1.12.1 Unfolded Protein Response (UPR)

Upon accumulation of unfolded and misfolded proteins, UPR is activated in order to upregulate ER chaperones. The increased number of chaperones is able to re-process the proteins that were unfolded or misfolded. The ER stress transducer proteins, inositol requiring-1 (IRE-1), activating transcription factor-6 (ATF6), and PKR-like ER kinase (PERK) are activated. IRE1 and PERK are released from GRP78 (Glucose regulated protein 78 kDa)/BIP (Immunoglobulin binding protein). ATF6 is activated by a two-step cleavage by Site-1 protease (S1P) and Site-2 protease (S2P). IRE1 initiates XBP-1 to activate the transcription of target genes for molecular chaperones and accessory proteins such as catalysts. ATF6 and PERK initiate the transcription factors, p50ATF6 and ATF4, respectively, which activate transcript of additional molecular chaperones and catalysts (68, 70, 71).

1.12.2 Translational attenuation

Translational attenuation lowers the load of client proteins by halting protein translation, thus giving the cell more time to process the unfolded proteins. PERK induces phosphorylation of eukaryotic initiation factor-2a (eIF-2a), which blocks translation initiation (70, 71).

1.12.3 ER associated degradation (ERAD)

ERAD is the pathway in which misfolded are designated for degradation. Unsalvageable proteins are transported to the proteasome by molecular chaperones. Misfolded proteins are transported out of the ER by GRP78 through the Sec61 complex to the cytosol. The misfolded proteins are ubiquitinated marking them for degradation by the 26S proteasome (70, 71).

1.12.4 ER stress-apoptosis

If a cell is unable to deal with the unfolded proteins, the ER stress pathway will initiate programmed cell death, or apoptosis (72). There are three known apoptosis pathways triggered by ER stress: (1) CHOP(C/EBP homologous protein)/GADD153, pathway, (2) cJUN NH2-terminal kinase (JNK) pathway, and (3) caspase-12 pathway. CHOP is the transcription factor that regulates one of the pathways. CHOP levels are temporally regulated from limited detection to strong expression upon ER stress activation. CHOP promotes apoptosis in response to ER stress as determined by overexpression and targeted disruption of the CHOP gene (70, 73, 74). It is not known what the exact targets of CHOP are that result in apoptosis, however, Marciniak et al demonstrate that CHOP acts on GADD34 to promote protein synthesis and alter the oxidation balance in the cell leading to ERO1 (ER oxidase 1) activation (75).

The JNK pathway is another means that leads to ER stress-apoptosis. JNKs are signal transduction proteins that regulate gene expression and participate in apoptosis/survival pathways in response to stressors. Upstream of JNK activation, the ER stress protein, Ire1 recruits TRAF2 and ASK1. The three proteins form a complex to activate JNK to induce apoptosis (70, 73).

The final ER stress-apoptosis pathway is through caspase activation, specifically caspase-12 in rodents (73, 76). Caspase-12 is activated by m-calpain (77), IRE1a/TRAF2 (78), or caspase-7 (79). Death receptor-mediated or mitochondria-targeted apoptotic signals are not responsible for caspase-12 activated when stimulated by ER stress (70, 73).

1.13 ER STRESS AND THE BETA CELL

Secretory cells, like islet beta cells and plasma cells, have a more developed ER in order to manage the continuous pressure of protein processing, which leads to continuous ER stress, due to upregulation of chaperones and accessory proteins required to maintain ER homeostasis (80). In islet beta cells, disequilibrium in the ER leading to activation of ER stress occurs in response to a number of cellular events. There is increasing evidence that cytokines, reactive oxygen species (ROS), viral infection, and drug/toxin exposure results in activation of ER stress. ROS has been shown to be important in beta cell death in T1D. Oyadomari et al demonstrated that MIN6, a mouse insulinoma cell line, produces nitric oxide (NO) in response to treatment with a cocktail of IL-1β, TNF-α, and IFN-γ cytokines. The production of NO correlates with the activation of ER stress, signified by the upregulation of GRP78, IRE1α, and PERK (74).

1.14 ER STRESS AND DISEASE

The role of ER stress in the pathogenesis of various diseases has begun to be elucidated. 'Conformational diseases' or 'folding diseases' are diseases caused by the misfolding of cellular protein causing aggregates that can initiate ER stress. Neurodegenerative diseases, bipolar disorder, T1D, T2D, atherosclerosis, ischemia, heart disease, liver disease, kidney disease, and other inflammatory diseases have shown to alter ER stress response. During T1D, inflammation plays a critical role in disease progression and, in some instances, is associated with ER stress. During inflammation NO-induced apoptosis, mediated by ER stress and CHOP, has been documented in beta cells (70, 81, 82) (69, 75). In the inflammatory disease, rheumatoid arthritis, GRP78 is a target autoantigen for T and B cells. Blass et al. reported that 63% of the 400 RA patients tested for T cell reactivity to GRP78 demonstrated specific T cell reactivity, when challenged with biochemically purified GRP78 from mammalian cells, in addition to GRP78 autoantibodies (83).

2.0 HYPOTHESIS AND SPECIFIC AIMS

It is long been proposed that there are environmental triggers that initiate the antigen-driven autoimmunity, which is responsible for the loss of beta cell mass in Type 1 Diabetes (T1D). A number of the environmental triggers are associated with T1D, however, there does not appear to be a common link between these environmental triggers. **I hypothesize that identification of additional autoantigens will yield a greater understanding of the pathogenesis of T1D specifically the antigen for the BDC-2.5 T cell clone.**

Specific Aim 1: Based on the concept of linked recognition, I will utilize the novel technique Restricted Immune System via Adoptive Transfer (RISAT) to drive an antibody response to the cognate antigen for the BDC-2.5 T cell clone.

Specific Aim 2: Using the antibodies from the BDC-2.5 RISAT mice, I will identify candidate antigens for the BDC-2.5 T cell clone. Proteomic analysis will be used to identify the protein(s) recognized by the RISAT antibodies.

Specific Aim 3: BDC-2.5 T cell assay will be preformed with candidate antigens to confirm their antigenicity to the BDC-2.5 T cell clone. The source of antigen will be from purified preparations or through in vivo upregulation in the non-antigenic NIT-1 cells.

3.0 MATERIALS AND METHOD

3.1 ANIMALS

BALB/c, DO11.10, CB17.*scid*, NOD, BDC-2.5/NOD TCR-Tg, and NOD.*Rag*$^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME) were housed and bred under specific pathogen-free conditions in the animal Facility of the Rangos Research Center at the University of Pittsburgh. BALB/c, DO11.10, CB17.*scid* mice were used to develop the DO11.10 Restricted Immune System via Adoptive Transfer (RISAT) mouse model. NOD and NOD.*Rag*$^{-/-}$ mice were used for the BDC-2.5 RISAT mouse model. BDC-2.5/NOD TCR-Tg mice were used to collect serum. Blood was collected for sera as approved by the University of Pittsburgh IACUC.

3.2 T- AND B-CELL ISOLATION, T CELL CLONE CULTURE, AND ANTIGEN-SPECIFIC T CELL ASSAY.

T-cells from DO-11.10 mice and B cells from BALB/c and NOD mice were purified from spleens by negative selection using MACS magnetic beads. The T and B cell purity was greater

than 95% (data not shown). BDC-2.5 T-cell clones were used after restimulation in culture with antigen/APC and further expansion in subculture with additional IL-2 as previously described (84). BDC-2.5 T cell clones were stimulated every 2 weeks with beta cell antigen and irradiated NOD splenocytes were used as APCs in complete media (62, 85). The antigen was in the form of a βmem made from beta-cell adenomas (62).

For antigen-specific assays, BDC-2.5 T cell clones were cultured in 96-well flat-bottom plates at a density of 2 x 10^4 cells/well, with or without 5 x 10^5 irradiated NOD splenocytes as APC and a source of antigen, such as untreated and thapsigargin treated NIT-1 cells (see below, under **Induction and Detection of ER stress and/or Apoptosis in Cell Lines**), or recombinant GRP78 (Stressgen; Victoria, BC) at 100μg/ml or 62.5μg/ml. The assay plates were incubated at 37°C for 72 hrs before the supernatants were harvested. IFN-γ production by the BDC-2.5 T cells was assessed by sandwich enzyme-linked immunosorbent assay (ELISA).

3.3 RISAT RECONSTITUTION VIA ADOPTIVE TRANSFERS, IMMUNIZATION, AND SERA COLLECTION.

CB17.*scid* mice were injected retro-orbitally with 2 x10^7 DO11.10 T-cells, followed by purified BALB/c B cells (2x10^7) 72 hrs later. The immunization agents were either whole OVA or as a negative control, pigeon cytochrome c (PCC). CB17.*scid* mice were immunized with 300μg OVA or 100μg PCC in Complete Freund's Adjuvant (CFA) into the base of the tail. On day 14, CB17.*scid* mice were re-immunized with 150μg OVA or 50μg PCC in Incomplete Freund's Adjuvant (IFA), and an additional boost of 100μg OVA or 100μg PCC in IFA was administered at day 53. "Pre", "post", "1st", and "2nd" immunization sera were collected and on day 58, a

post-"3rd" bleed was collected. The resultant sera was screened by Western blot for immunoglobulin production after each treatment.

Adult NOD.*Rag*$^{-/-}$ mice were pre-bled prior to receiving 2x10^7 BDC-2.5 T cells (day -7) retro-orbitally. Control animals received HBSS only. On day -5 all NOD.*Rag*$^{-/-}$ animals received purified NOD B cells (2x10^7) by retro-orbital injection. On day 0, serum was again collected from all animals by centrifugation (14,000 x g) of peripheral blood, and the reconstituted NOD.*Rag*$^{-/-}$ mice were immunized with 100µg of a βmem derived from beta cell adenoma cells as previously described (62), in CFA in the base of the tail (day 0). On day 14, a post-"1st" immunization bleed was collected, and all mice were boosted with 50µg of βmem in IFA (day 14). On day 21, a post-"2nd" boost bleed was collected.

3.4 IMMUNOBLOT ANALYSIS

50µg, unless otherwise noted, of protein was separated on a 12% SDS-PAGE prep gel using Mini-Protean II electrophoresis (Bio-Rad, Hercules, CA) at 150V for 1 h. Proteins were transferred onto 0.45μm charged polyvinylidene fluoride (PVDF) membranes using a Mini Trans-Blot electrophoretic transfer apparatus (Bio-Rad) at 100V for 1 hr at 4°C in Transfer Buffer (containing 25mM Tris-HCl, 192mM glycine, 10% methanol, pH=8.3). Membranes were stained with Ponceau S stain (0.1% (w/v) in 1.5% trichloracetic acid (v/v)) to visualize protein bands and rinsed with 40% methanol and 7.5% glacial acetic acid to remove excess stain. Membranes were blocked in 5% milk in PBS containing 0.05% Tween-20 (PBST) for 1 hr at room temperature. Primary antibodies, commercially available or sera, were diluted in 5% BSA

in PBST, then incubated with membranes overnight at 4°C. Membranes were probed with secondary antibodies diluted in 5% milk in PBST and bands were detected with ECL Plus reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's protocol. Phosphoserine (P-ser), phosphotyrosine (P-tyr) and phosphothreonine (P-thr) were detected with Sigma phospho-specific antibodies.

3.5 SCREENING SERUM FOR AUTOANTIBODIES

Sera from RISAT reconstituted CB17.*scid*, NOD.*Rag*$^{-/-}$, NOD (2 months non-diabetic, 4 months diabetic, and 6 months diabetic), and NOD.BDC-2.5 TCR-Tg mice were screened for antibodies against either OVA or βmem. The screening method was similar to immunoblot analysis described in "**Immunoblot Analysis**" except for the following steps. 50μg OVA or βmem were separated on a 12% SDS-PAGE prep gel and then transferred onto PVDF membranes. Membranes were blocked in 5% milk in PBST for 1 hr at room temperature. The membrane was placed into the Mini-Protean II Multiscreen Apparatus (Bio-Rad). Sera from the RISAT-treated, NOD or NOD.BDC-2.5 TCR-Tg mice were diluted in 5% BSA in PBST and added into a specific slot of the Multiscreen blotter, then incubated overnight at 4°C. After the slots were flushed with PBST, membranes were removed from the apparatus and probed with goat anti-mouse HRP secondary antibody and bands were detected with ECL Plus reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's protocol.

3.6 IMMUNOPRECIPITATION OF AUTOANTIGENS

50μg of βmem, NIT-1 untreated or treated with thapsigargin (1μM or 5μM) or HeLa untreated or treated with thapsigargin (1μM or 5μM) lysate was incubated with 50μl NOD.BDC-2.5 TCR-Tg mouse sera overnight at 4°C. The following day, 25μl of washed Protein G Sepharose (Sigma) was incubated with the protein:antibody complexes for 2 hrs at 4°C. The immunoprecipitates were collected by centrifugation at 540x g, washed twice with RIPA (Radio-Immunoprecipitation Assay) Buffer (containing 1% Nonidet P-40 or Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (1x, Roche, Nutley, NJ) in PBS), and then heated in Laemmli buffer at 100°C for 5 min. The immunoprecipitated proteins were separated on 4-20% gradient SDS-PAGE gels. Immunoblot analysis was preformed as described in "**Immunoblot Analysis**".

3.7 PROTEOMIC ASSAYS FOR IDENTIFICATION OF CANDIDATE AUTOANTIGEN.

For MALDI-ToF, protein bands on a 4-20% gradient gel were stained with GelCode® Blue reagent (Pierce Biotechnology, Rockford, IL). The Proteomics Core Lab at the University of Pittsburgh preformed MALDI-ToF analysis on bands of interest. For Edman analysis, protein bands were transferred onto 0.45μm charged PVDF membranes using a Mini Trans-Blot electrophoretic transfer apparatus (Bio-Rad) at 100V for 1 hr at 4°C in Transfer Buffer. Membranes were stained with GelCode® Blue reagent to visualize protein bands. The

Laboratory of John Hempel (Department of Biological Sciences, University of Pittsburgh) preformed Edman degradation on bands of interest.

3.8 INDUCTION AND DETECTION OF ER STRESS AND/OR APOPTOSIS IN CELL LINES.

NIT-1 cells (passage 27) (86) (ATCC, Manassas, VA) were propagated in 75-cm^2 flasks at 37°C in culture media (CM). Cells were fed with new medium every other day and were grown to confluence in the tissue culture flasks, at which time they were harvested using non-enzymatic Cell Dissociation Buffer (Invitrogen, Carlsbad, CA) and transferred to the appropriate culture dishes, either for expansion or for the described experiments. To induce ER stress and/or apoptosis, cells were treated with 1 or 5μM thapsigargin for 60 min or 10, 25 or 50μM *N,N,N′,N*-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN) for 4 hr and then washed with CM three times. Cells were centrifuged and collected for biochemical assays after two washes with PBS or transferred to 96-well plates for T cell assays.

Induction of ER stress was identified by immunoblotting and detecting GRP78 levels. 50μg of NIT-1 or HeLa lysates in RIPA buffer (containing 1% Nonidet P-40 or Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail (1x, Roche, Nutley, NJ) in PBS) were separated on a 4-20% gradient SDS-PAGE gel. Immunoblot analysis was followed as described in "**Immunoblot Analysis**". The following primary antibodies were used anti-BiP/GRP78 antibody (Stressgen; Victoria, BC, 1:1000; Santa Cruz (N-20), Santa Cruz (C-20), or Santa Cruz (H129); Santa Cruz, CA, 1:1000), or anti-beta actin antibody (Sigma; 1:15,000) in 5% BSA in PBST. Goat anti-rabbit, rabbit anti-goat and goat anti-mouse (1:15,000) IgG

polyclonal antibody conjugated to HRP were used as secondary antibodies.

In order to identify the NIT-1 cells as apoptotic or necrotic after treatment with thapsigargin or TPEN, a quantitative fluorescent assay was performed as previously described (84). Cells were harvested and transferred to a microcentrifuge tube and centrifuged for 5 min at 200g. Supernatants were aspirated carefully, leaving 25 µl for resuspension of the cell pellets by gentle shaking of the tube. After addition of 2 µl of DNA intercalating dye mix (100 µg/ml acridine orange & 100 µg/ml of ethidium bromide in PBS), 10 µl of the NIT-1 cell suspension was transferred to a clean microscope slide, and a coverslip was placed on the suspension. Using a fluorescence microscope with a FITC/TRITC filter, cells were scored for morphological evidence of apoptosis versus necrosis by visualization of the cell for both the color and the state of the nucleus, using a mixture of the DNA intercalating dyes acridine orange and ethidium bromide. The criteria we used to score the cells after treatment were as follows: 100 cells (minimum) were scored into one of four categories: Viable cells with normal nuclei (green nuclei), necrotic cells with normal nuclei (orange nuclei), apoptotic cells with intact membranes (highly condensed green nuclei), and apoptotic cells with damaged membranes (highly condensed orange nuclei). The percentages of the morphological categories were then calculated.

3.9 GRP78 PEPTIDE LIBRARY

Overlapping 15mer peptides of murine GRP78 (ID P20029) were synthesis by Mimotope (Clayton Victoria, Australia). Peptides were pooled into groups of 12 or 13 peptides. Peptide pools were assessed at 2.8, 1.4, and 0.7ug of peptide per well in T cell clone assays as previously

described in "**T- and B-cell isolation, T cell clone culture, and antigen-specific T cell assay"**. Individual and surrounding peptides were assayed if they stimulated the T cells in both pools, i.e. p32, p33, p34, p35, p36, p37, p38.

3.10 ATP 8-AH SEPHAROSE PURIFICATION OF GRP78

GRP78 was selectively purified by JenaBioscience ATP 8-AH sepharose. NIT-1 cells treated with 5μM were lysed with Triton X-114 (87). Lysates were dialyzed against 20 mM Tris pH 7.5, 20 mM NaCl, 10 mM $MgSO_4$, 14 mM 2-ME at 4^0C overnight in a (0.5 – 3 mL) 10K MWCO slide-a-lyzer with several buffer exchanges. Dialyzed lysates were mixed with 1mL of ATP 8-AH sepharose and incubated overnight at 4^0C on a rocking platform. The columns were washed and ATP-binding proteins were eluted with 10-20mM ATP. Eluted proteins were analyzed by silver stain, immunoblot, and T cell assay as described in "**Immunoblot Analysis**" and "**T- and B-cell isolation, T cell clone culture, and antigen-specific T cell assay"**. Silver stain was preformed with Amersham Silver Stain Plus according to manufacture's protocol.

3.11 REMOVAL OF O-LINKED GLYCOSYLATION

NIT-1 cells were treated with 5μM thapsigargin. Lysates were prepared in RIPA buffer. To remove sites of O-linked glycosylation cells were treated as described in Mansour et al (88). Briefly, lysates were incubated with 0.1M NaOH at 37^0C for 0 and 3 hours. 0.1M glacial acetic acid was added to neutralize NaOH. Immunoblots and T cell assays were performed with lysates

as described in "Immunoblot Analysis" and "**T- and B-cell isolation, T cell clone culture, and antigen-specific T cell assay**". For immunoblot, anti-mouse O-linked N-acetylglucosamine antibody (CTD110.6, Abcam, Cambridge, MA; 1:1000) or Stressgen anti-GRP78 was used as primary antibody. Goat anti-mouse and goat anti-rabbit were used as secondary antibodies, respectively.

3.12 CITRULLINATION DETECTION ASSAY

To detect citrullinated protein in our lysates, we used the anti-citrulline (modified) detection kit (Upstate, Lake Placid, NY) according to manufacture's protocol. Protein samples were separated on a 4-20% gradient SDS-PAGE gel using Mini-Protean II electrophoresis (Bio-Rad, Hercules, CA) at 150V for 1 h. Proteins were transferred onto 0.45μm charged PVDF membranes using a Mini Trans-Blot electrophoretic transfer apparatus (Bio-Rad) at 100V for 1 hr at 4°C in 25mM Tris-Hcl, 192mM glycine, 10% methanol, pH=8.3. Membranes were modified overnight in water bath at 37ºC in a 50/50 mixture of Reagent A and B. Reagent A is 0.025% $FeCl_3$ in a solution of sterile, distilled water/98% H_2SO_4/85% H_3PO_4. Reagent B is 0.5% 2,3-butanedione monoxime, 0.25% antipyrine, 0.5M acetic acid. After 4 washes with water, membranes were then block in 3% Milk-TBS (TBS-MLK) for 30 minutes. Membranes were incubated for 30 minutes with Anti-citrulline (modified) antibody, which was diluted at 1:1000 in TBS-MLK. Two washes with water were followed with a 45 minutes incubation with goat anti-rabbit HRP conjugated IgG (1:5000). The membranes were then washed twice with water, followed by a wash with TBS-0.05% Tween-20 for 3 minutes. The membranes were washed 4 times with water before bands were detected with ECL Plus.

4.0 GLUCOSE REGULATED PROTEIN 78 (GRP78): A CANDIDATE ANTIGEN FOR THE DIABETOGENIC T CELL CLONE, BDC-2.5.

Adapted from manuscript. Sheila Schreiner*, Hubert M. Tse†, Gina M. Coudriet*, Jennifer L. Profozich†, Gene Barbour‡, Brenda Bradley‡, Kathryn Haskins‡ and Jon D. Piganelli*†2

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4.1 INTRODUCTION

Type 1 Diabetes (T1D) is initiated by T cell recognition of pancreatic beta cell antigens and is characterized by T cell-mediated leukocytic infiltrate into the pancreatic islets, beta cell destruction, and ultimately hyperglycemia. It has been well established in T1D patients and recapitulated in animal models of T1D that human HLA class II / mouse MHC II is a major determinant of susceptibility (55, 89). The importance of MHC class II is reinforced by the fact that diabetogenic $CD4^+$ T cells are absolutely critical for the initiation of T1D in open T cell repertoire animal models (85, 90). Thus knowledge gained, regarding self-antigen interaction

with these class II molecules will provide insight into the events that precipitate the breakdown of self-tolerance in T1D. The NOD mouse is a well-characterized model of T1D that closely resembles the human form of the disease.

To investigate the role of $CD4^+$ T cells and the autoantigens to which they respond in T1D, a panel of $CD4^+$ Th1 T cell clones recognizing islet beta cells as antigen and able to rapidly cause diabetes in very young (<10 day-old) NOD recipients was produced (61, 85). The properties of these T cell clones have been described in a number of publications and, in the case of one clone, BDC-2.5, a TCR-transgenic (91) model (92) has made possible a variety of *in vivo* studies (85). The $CD4^+$ BDC-2.5 T cell clone responds to antigen in the form of whole mouse islet cells, or membrane preparations from beta tumor cells (βmem), presented in the context of IA^{g7} (62, 93).

Although it has been 20 years since this clone was first reported, neither the natural peptide ligand nor the protein source of the peptide antigen has been identified. On the other hand, various peptide mimotopes that can stimulate activity of the BDC-2.5 clone or 2.5 TCR-Tg T cells have been reported. Judkowski et al described a series of peptides that were able to induce responses of varying magnitude in T cells from the 2.5 TCR-Tg mice (94). It was of note in this study that based on homology to a GAD peptide sequence, the authors speculated that GAD was the source of the natural antigen ligand for BDC-2.5. Since other studies, however, have failed to confirm any antigenic activity with GAD, or sequential peptides from GAD, with the BDC-2.5 T cell clone, it is doubtful that GAD is the source of antigen for this clone (85). Yoshida et al reported on several peptide mimotopes, identified through screening of a combinatorial peptide library, that activated clones from two panels of NOD-derived $CD4^+$ T cell clones isolated from two separate labs (95). These peptides behaved as agonists, eliciting strong responses from several clones in the panels. Since no homology could be found between

sequences in these mimotopes and peptides in data banks, proteins could not be identified as candidate antigens from this study.

Despite extensive investigation, the events that trigger T1D remain unknown. What is becoming evident in at risk individuals and in animal models is that events leading to the presentation of beta cell associated oligopeptide antigens to autoreactive T cells during the initiation of T1D may result from normal physiological actions during the development of beta cells or routine beta cell metabolic function. The production of neo-antigens, coupled to defective APC function may be a recipe for beta cell selective autoimmunity (96) (57, 58, 97). T1D, as manifested in the NOD model, has been described as a progression through a series of checkpoints. First there is the expansion of the autoreactive pool (checkpoint 1), followed by homing of the T cells to the pancreas (checkpoint 2), and finally the transition from peri-insulitis to invasive, destructive insulitis (checkpoint 3) (98). To better understand the initiating events and the role of $CD4^+$ T cells in the pathogenesis of T1D, I have utilized BDC-2.5 T cell clones, in a T cell:B cell model system, which I have termed Restricted Immune System via Adoptive Transfer (RISAT). It is well established that long-lived B cells circulate through the blood and lymph nodes until they encounter their specific antigen that has been ferried to the T cell areas of the lymph node or spleen by macrophages and/or dendritic cells. Interaction with antigen inhibits B cell migration and leads to BCR-specific internalization of antigen (99, 100). Naïve antigen-specific Th cells are also activated in these locations, by antigen-presenting dendritic cells. During T cell-dependent activation, an APC presents a processed antigen to a Th cell resulting in T cell priming. When a B cell processes and presents the same antigen to the primed Th cell, it leads to formation of stable T and B cell conjugates, termed linked-recognition, including the interaction of CD40L and CD40 on the T cells and antigen-specific B cells,

respectively (101). These signals induce B cells to proliferate and terminally differentiate into antibody-secreting cells. By using only the BDC-2.5 T cell clone to provide help to B cells after immunization with a previously described (62) antigenic beta cell membrane preparation (βmem) in NOD.*Rag*$^{-/-}$ mice, I was able to utilize linked recognition to drive antigen specific antibody production. The resultant serum contained an antibody that immunoprecipitated the ER-associated protein GRP78 within the βmem. This study suggests that the BDC-2.5 T cell recognizes the autoantigen, GRP78, during the normal physiological process of ER stress in pancreatic beta cells.

4.2 RESULTS

4.2.1 Proof of concept: RISAT with the DO11.10 T cell.

To test the RISAT model, the OVA-specific DO11.10 T cell receptor transgenic mouse model was utilized (102). CB17.*scid* mice, which lack T cells and mature B cells, were reconstituted with DO11.10 T-cells and BALB/c B-cells, followed 10 days after transfer, by immunization with either OVA or the non-related antigen PCC (**Figure 4.1**). Again, by reconstituting the *scid* mice with a single T cell clone, an open repertoire of B cells and then immunizing the mice with their cognate antigen, through linked recognition the T cells should lend help to the B cells to produce IgG antibodies against the cognate antigen.

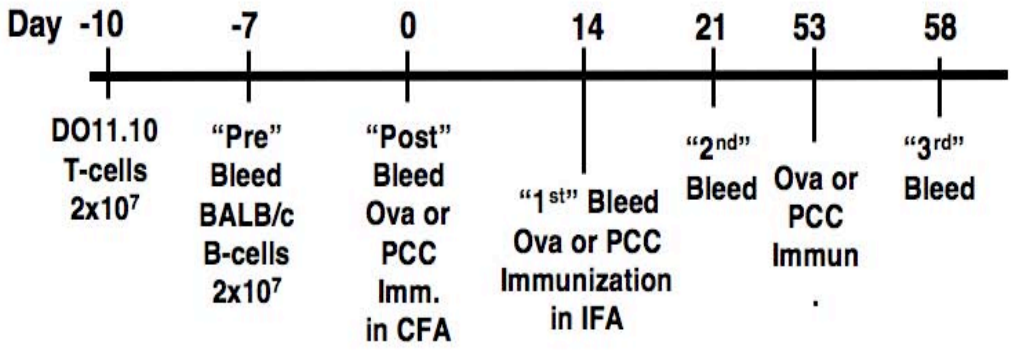

Figure 4.1 Timeline of transfers and immunization schedule for DO11.10 RISAT.

Sera from the DO11.10 RISAT mice were collected prior to and after each immunization, and then screened for antibody production by Western blot analysis. Antibody production in the DO11.10 RISAT model was detected after the first boost of whole OVA using a secondary antibody that is specific for the light chain of mouse IgG, and was absent prior to the adoptive transfer of DO11.10 T-cells and BALB/c B-cells and, before immunization. Animals immunized with the irrelevant antigen PCC did not produce IgG light chain, supporting the linked recognition strategy (**Figure 4.2**). Furthermore, animals immunized with OVA generated only OVA-specific antibodies that had no specific cross-reactivity to PCC (**Figure 4.3**).

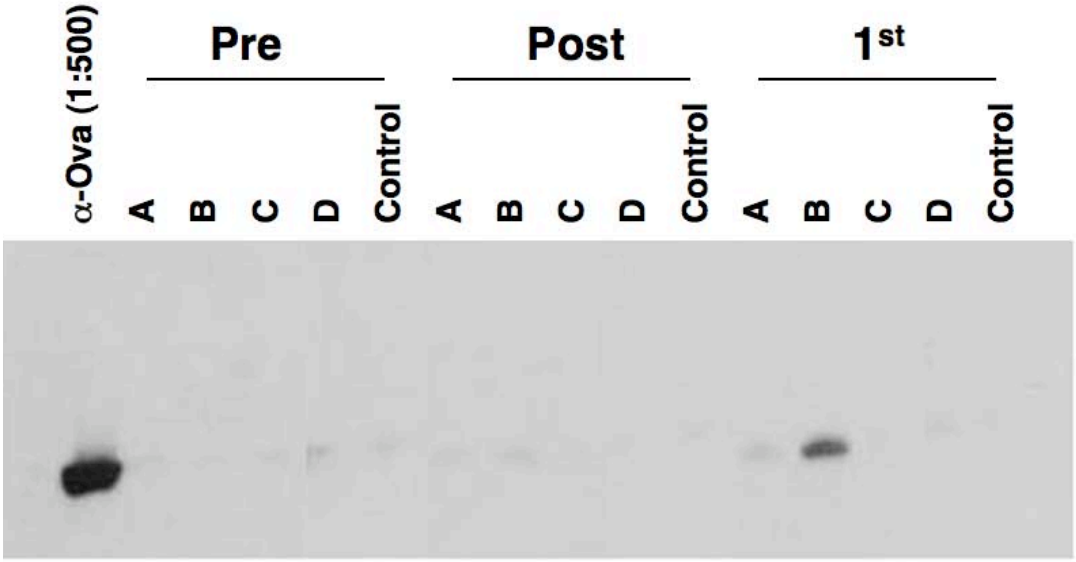

Figure 4.2 Screening DO11.10 RISAT mouse serum for antibody production. To screen DO11.10 RISAT mouse serum for antibody production, sera from four mice (A-D) were run on a gradient gel and transferred to a PVDF membrane. The membrane was probed with an anti-mouse antibody to determine which mice (1-4) were producing antibodies after transfer of DO11.10 T-cells and BALB/c B-cells and immunization with whole OVA. Control mice received DO11.10 T-cells and BALB/c B-cells and were immunized with PCC (pigeon cytochrome-c). Pre, before adoptive transfers and immunization; Post, after adoptive transfers of T-cells and B-cells, before immunization; 1st, after adoptive transfers and 1st immunization.

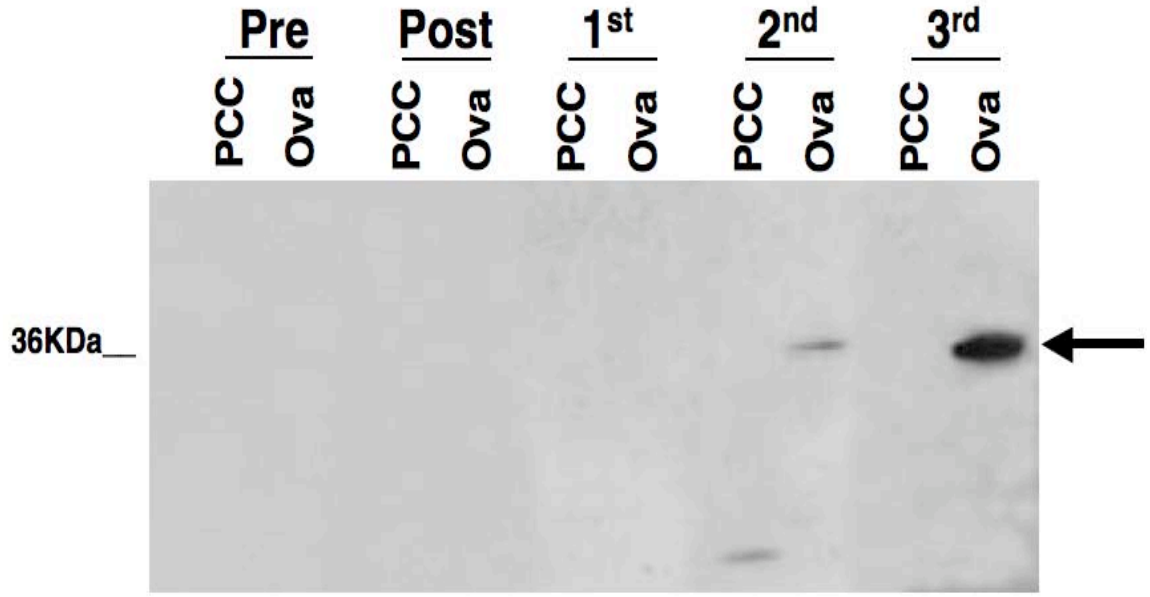

Figure 4.3 DO11.10 RISAT generated serum recognizes its cognate antigen, OVA. 50μg of whole OVA or PCC was immunoblotted against DO11.10 RISAT generated serum. Mouse B serum was positive for antibody production; thus it was used for the immunoblot. Pre, before adoptive transfers and immunization; Post, after adoptive transfers of T-cells and B-cells, before immunization; 1st, after adoptive transfers and 1st immunization. 2nd, after 2nd immunization; 3rd, after 3rd immunization.

4.2.2 RISAT-generated sera from NOD.*Rag*$^{-/-}$ mice immunoprecipitates GRP78 from βmem.

The results of the DO11.10 RISAT experiment demonstrated that the RISAT system for antibody generation based on linked recognition would work. After NOD.*Rag*$^{-/-}$ mice were populated with BDC-2.5 T cell clones and NOD derived B cells, I detected increased antibody production with each immunization with of βmem preparation (**Figure 4.4**). There was antibody production present in the control lane, however, these antibodies were not specific to βmem (data not shown).

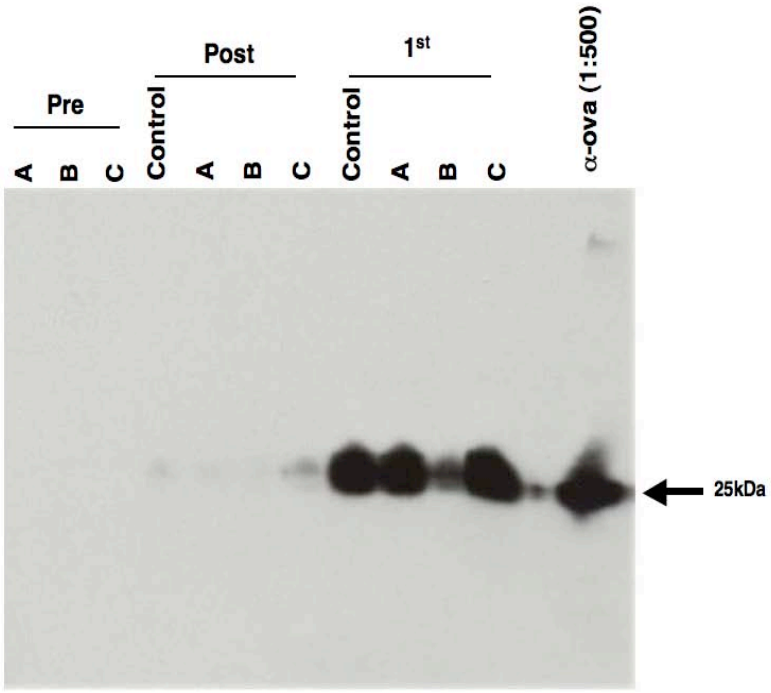

Figure 4.4 Screening for antibody production in the BDC-2.5 RISAT mice. To screen the BDC-2.5 RISAT generated serum for antibody production; sera were run on a gel and transferred to a PVDF membrane. The membrane was probed with an anti-mouse antibody in order to determine if the BDC-2.5 RISAT mice (A, B, and C) were producing antibodies after reconstitution with both BDC-2.5 T cells and NOD B cells and immunization with βmem. Control sera are from mice that received only NOD B cells and immunized with βmem.

Sera from RISAT mice were screened for antibody production against antigenic βmem and non-antigenic NIT-1 lysate. Control mice did not produce antibodies that recognized protein in either βmem or NIT-1 lysate (data not shown). Serum from BDC-2.5 RISAT mouse A produced βmem specific antibodies. Therefore, it was used to perform a traditional immunoblot with NIT-1 lysates and βmem to determine which protein(s) were recognized by the immune serum. The serum recognized a protein at molecular weight 70kDa in the βmem fraction, but failed to respond strongly to any proteins in the NIT-1 lysate (**Figure 4.5**).

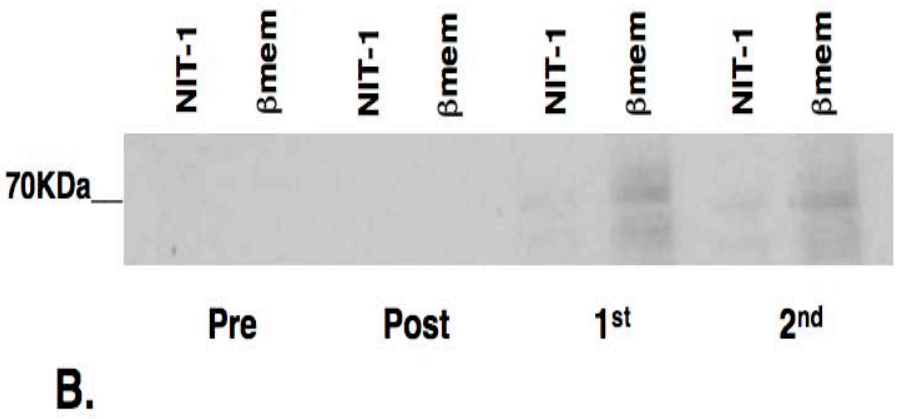

Figure 4.5 Immunoblot of NIT-1 lysate and βmem with BDC-2.5 RISAT serum. NIT-1 lysate and βmem were separated by SDS-PAGE gel and transferred to a PVDF membrane. The membrane were probed with BDC-2.5 RISAT serum from mouse A which was collected at various timepoints, pre., post, 1st, and 2nd. Serum was diluted at 1:200.

Table 4.I Identification of 70kDa protein band with MALDI-ToF.

Observed	Mr (expected)	Mr (calculated)	Δdaltons	Amino Acid position		Peptide Sequence
				Start	End	
1191.67	1190.67	1190.63	0.04	466	475	VYEGERPLTK
1210.63	1209.62	1209.58	0.04	378	387	EFFNGKEPSR
1316.69	1315.68	1315.68	0.00	523	533	NKITITNDQNR
1329.70	1328.69	1328.61	0.08	328	337	FEELNMDLFR
1397.84	1396.83	1396.78	0.05	623	634	ELEEIVQPIISK
1430.73	1429.72	1429.68	0.04	103	114	TWNDPSVQQDIK
1460.81	1459.80	1459.75	0.05	355	368	SDIDEIVLVGGSTR
1512.80	1511.80	1511.74	0.05	326	337	AKFEELNMDLFR
1528.80	1527.79	1527.74	0.05	326	337	AKFEELNMDLFR
1536.85	1535.84	1535.79	0.05	140	153	TFAPEEISAMVLTK
1552.84	1551.83	1551.79	0.04	140	153	TFAPEEISAMVLTK
1566.84	1565.83	1565.77	0.05	62	75	ITPSYVAFTPEGER
1588.92	1587.91	1587.85	0.06	354	368	KSDIDEIVLVGGSTR
1604.90	1603.90	1603.86	0.04	125	139	TKPYIQVDIGGGQTK
1659.92	1658.92	1658.89	0.03	199	214	IINEPTAAAIAYGLDK
1677.87	1676.86	1676.80	0.06	83	97	NQLTSNPENTVFDAK
1816.05	1816.05	1814.99	0.06	199	215	IINEPTAAAIAYGLDKR
1836.99	1835.99	1835.93	0.06	449	465	SQIFSTASDNQPTVTIK
1888.04	1887.03	1886.96	0.07	166	182	VTHAVVTVPAYFNDAQR
1903.98	1902.98	1903.12	-0.14	1	18	MMKFTVVAAALLLLGAVR
1934.08	1933.07	1933.01	0.07	476	493	DNHLLGTFDLTGIPPAPR
1974.98	1973.98	1973.90	0.08	603	618	IEWLESHQDADIEDFK
1999.16	1998.15	1998.08	0.07	494	511	GVPQIEVTFEIDVNGIIR
2016.16	2015.15	2015.06	0.09	165	182	KVTHAVVTVPAYFNDAQR
2149.07	2148.06	2147.99	0.07	308	325	IEIESRREGEDFSETLTR
2174.13	2173.12	2173.03	0.09	603	620	IEWLESHQDADIEDFKAK
2178.07	2177.07	2176.96	0.10	635	655	LYGSGGPPPTGEEDTSEKDEL

Band immunoprecipitated and visualized with Gel Code Blue® in Figure 4.6A and later identified in Figure 4.6B as GRP78 by immunoblot was excised from SDS-PAGE gel stained with Gel Code Blue®. MALDI-ToF was preformed on excised band. Band was identified as GRP78 with a score of 191 with complete coverage using Matrix Science Mascot database-searching program.

The BDC-2.5 RISAT generated serum was used in an immunoprecipitation assay with the βmem preparation. Immunoprecipitated proteins were subjected to SDS-PAGE and then visualized by GelCode® Blue staining (**Figure 4.6A)**. A 70kDa band present on the gel was subjected to MALD-TOF analysis. MALDI-TOF spectrometry and analysis of the peaks via the Matrix Science Mascot database-searching program determined the protein to be GRP78 with a high degree of probability **(Table 4.I).**

The results from the MALDI-ToF analysis were also confirmed by Edman degradation with immunoprecipitated βmem protein with BDC-2.5 RISAT generated serum (data not shown). To demonstrate that the BDC-2.5 RISAT produced sera contained generated antibodies specific to GRP78, they were used as primary antibodies in an immunoprecipitation reaction with βmem protein. The resultant immunoprecipitated protein was transferred to a PVDF-membrane and probed with a commercially available antibody to GRP78 via immunoblot **(Figure 4.6B)**.

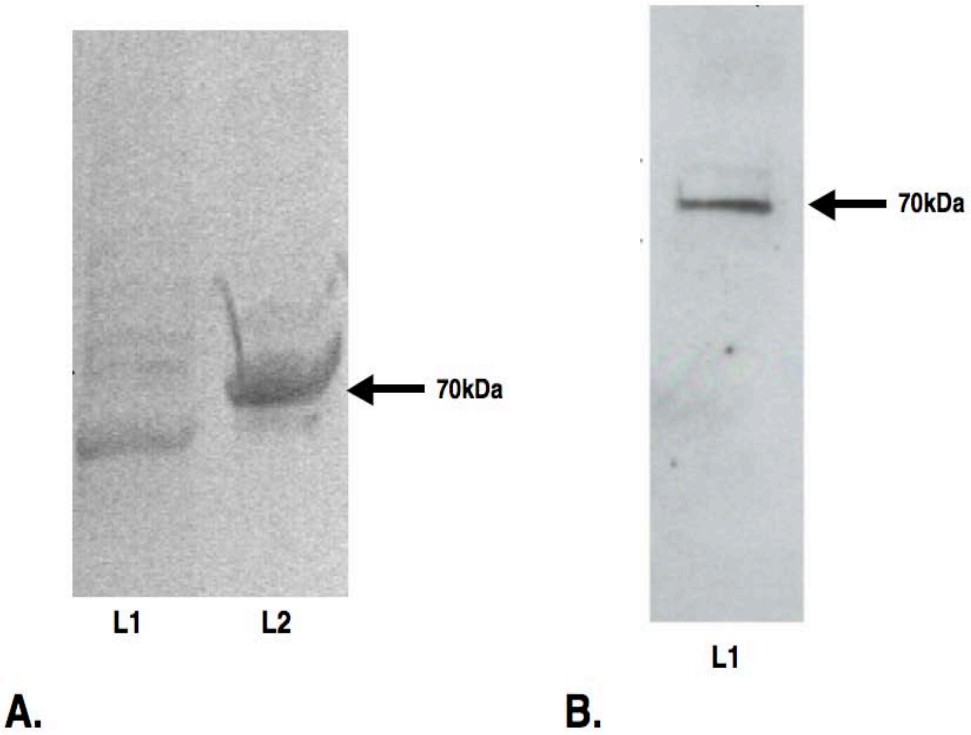

Figure 4.6 Immunoprecipitation to confirm that BDC-2.5 RISAT serum recognizes GRP78. A) Protein bands were visualized with Gel Code Blue®. Lane 1 (L1): βmem, L2: Immunoprecipitated βmem. For immunoprecipitation, βmem was incubated with BDC-2.5 RISAT generated serum. Protein:antibody complexes were pulled down with Protein G and separated on a SDS-PAGE gel and stained with Gel Code Blue®. B) Immunoblot of immunoprecipitated βmem. Immunoprecipitated βmem was run out on a SDS-PAGE gel and transferred to a PVDF membrane, blocked and probed with Stressgen anti-GRP78 antibody. After incubation with an anti-rabbit antibody, a signal was detected using ECL Plus reagent.

4.2.3 NOD and NOD.BDC-2.5 TCR-Tg mouse sera contain autoantibodies to GRP78.

To confirm the immunoprecipitation results achieved with the RISAT-produced serum, I analyzed serum from different aged NOD and NOD.BDC-2.5 TCR-Tg mice to determine if they inherently produced autoantibodies to GRP78. **Figure 4.7A** demonstrates that serum from female NOD mice display autoantibodies against numerous βmem proteins including GRP78 as early as 2 months of age. This pattern of antibody reactivity would be expected since the standard NOD mouse has a full T and B cell repertoire that could potentially allow T cell reactivity to give help to a multitude of B cells in generating autoantibody synthesis. Interestingly, when I used serum from NOD.BDC-2.5 TCR-Tg mice as a primary antibody against βmem protein in a Western blot, autoantibody specificity against GRP78 was detected in these mice (**Figure 4.7B**). These results demonstrate that both NOD and NOD.BDC-2.5 TCR-Tg mice are capable of producing autoantibodies to GRP78, further confirming the relevance of the BDC-2.5 RISAT results and the potential for ER stress induction of autoantigen synthesis.

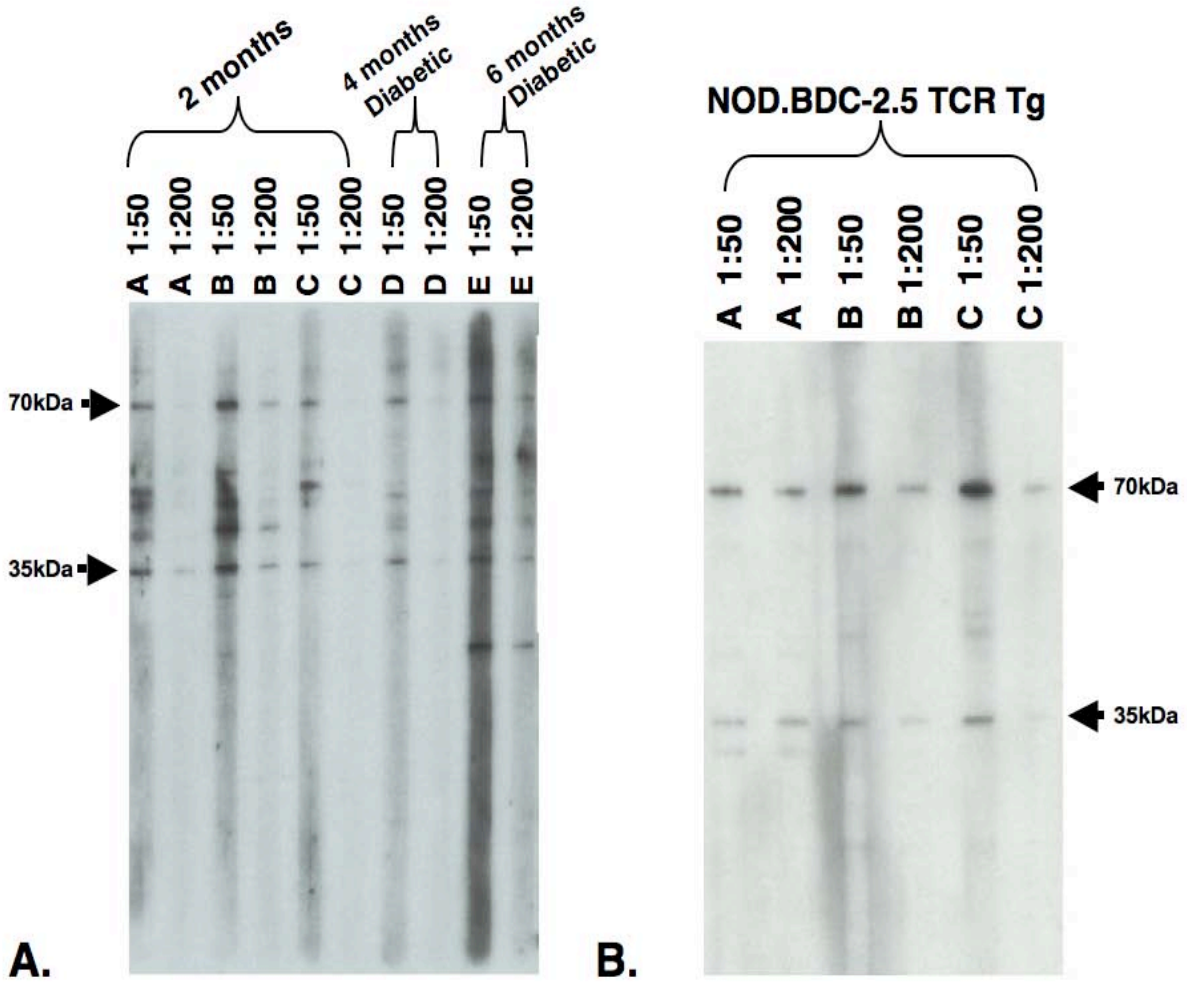

Figure 4.7 NOD and NOD.BDC-2.5 TCR Tg confirm anti-GRP78 results with BDC-2.5 RISAT generated serum. A) Serum from NOD mice at 2, 4, and 6 months and B) serum from three different NOD.BDC-2.5 TCR Tg mice were screened for anti-GRP78 antibodies. Letter designations above lanes represent individual mice. For both panels, 50μg of βmem were run out on a SDS-PAGE preparative gel and transferred to a PVDF membrane. Serum samples were diluted at 1:50 and 1:200 with 5% BSA in PBST and incubated overnight. Then the membranes were incubated with an anti-mouse secondary antibody, which was detected with ECL Plus reagent.

4.2.4 *E.coli* expressed recombinant GRP78 did not elicit IFN-γ production from the BDC-2.5 T cell clone.

To confirm whether the BDC-2.5 T cell recognizes GRP78, commercially available recombinant GRP78 produced in *E.coli* was assessed for antigenicity in an *in vitro* T cell assay using the NOD.BDC-2.5 TCR-Tg mouse derived T cells. Unexpectedly, the recombinant GRP78 failed to elicit a detectable IFN-γ response from NOD.BDC-2.5 TCR-Tg mouse derived T cells (data not shown), however, protein expression in *E.coli* differs from mammalian cells. Hence, these differences, i.e. posttranslational modification of proteins, may alter the antigenic epitope of GRP78.

4.2.5 Increased GRP78 production induced by ER stress, but not apoptosis, in the beta cell adenoma line NIT-1 and non-beta cell line HeLa enhances IFN-γ expression by BDC-2.5.

The results suggest that the BDC-2.5 T cell responds to a GRP78 peptide *in vivo* and supports the production of GRP78-specific autoantibodies, but the clone does not react to a recombinant form of GRP78 *in vitro*. As GRP78 is a key protein involved in the regulation of ER stress and since pancreatic beta cells are subject to secretory cell induced ER stress that results in a higher level of ER stress (73, 80, 103), I decided to test whether induction of ER stress in a beta cell insulinoma would lead to the necessary modification of the GRP78 and confer antigenic recognition for the BDC-2.5 T cell. ER stress was induced in NIT-1 cells with thapsigargin to increase GRP78 synthesis. Thapsigargin is an irreversible inhibitor of the ER Ca^{2+}-ATPase

(104), which leads to elevated cytosolic calcium concentration within the cell, and ultimately leads to ER stress.

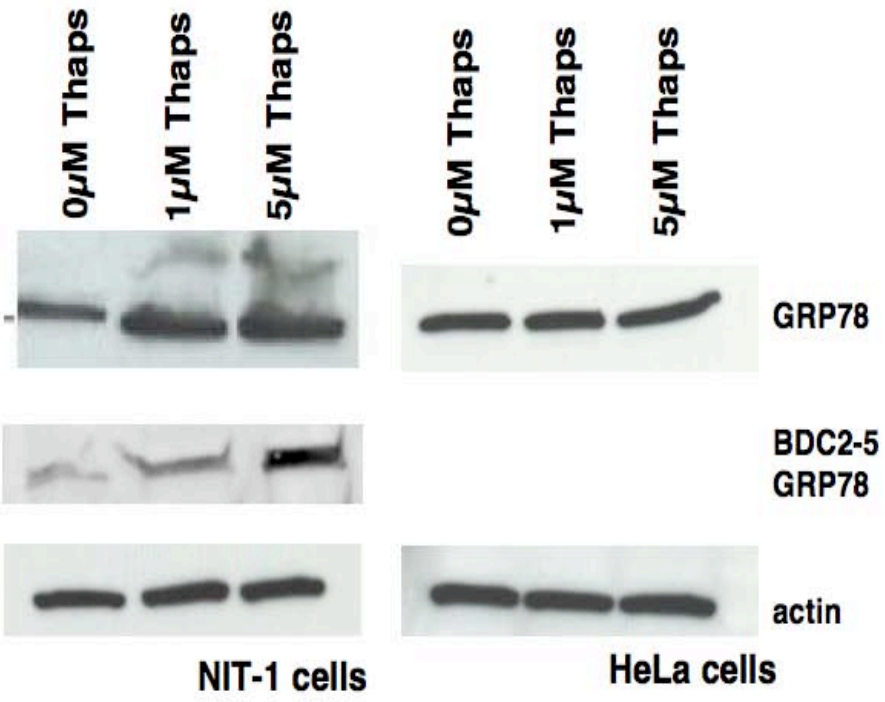

Figure 4.8 Upregulation of GRP78 in NIT-1 and HeLa cells when treated with thapsigargin to induce ER stress. NIT-1 and HeLa cells were treated for 60 min with 0, 1, or 5μM thapsigargin. Cells were lysed with RIPA buffer. 50μg of lysate was separated on a SDS-PAGE gel and transferred to a PVDF membrane, blocked and probed with anti-GRP78 or anti-beta actin (as a loading control) antibody. For NIT-1 cells, the Stressgen anti-GRP78 antibody was used. For HeLa cells, the Santa Cruz anti-GRP78 (N-20) antibody was used as it recognizes human GRP78. After the incubation with the secondary antibody, bands were detected using ECL Plus reagent. For detection of BDC-2.5 GRP78, 50μg of the above mentioned lysate was immunoprecipitated with 50μl of BDC-2.5 TCR Tg serum. Protein:antibody complexes were pulled down with Protein G and was separated by SDS-PAGE gel. After transferring to a PVDF membrane, the membrane was probed with Santa Cruz anti-GRP78 (C-20) antibody. After incubation with the secondary antibody, bands were detected using ECL Plus reagent.

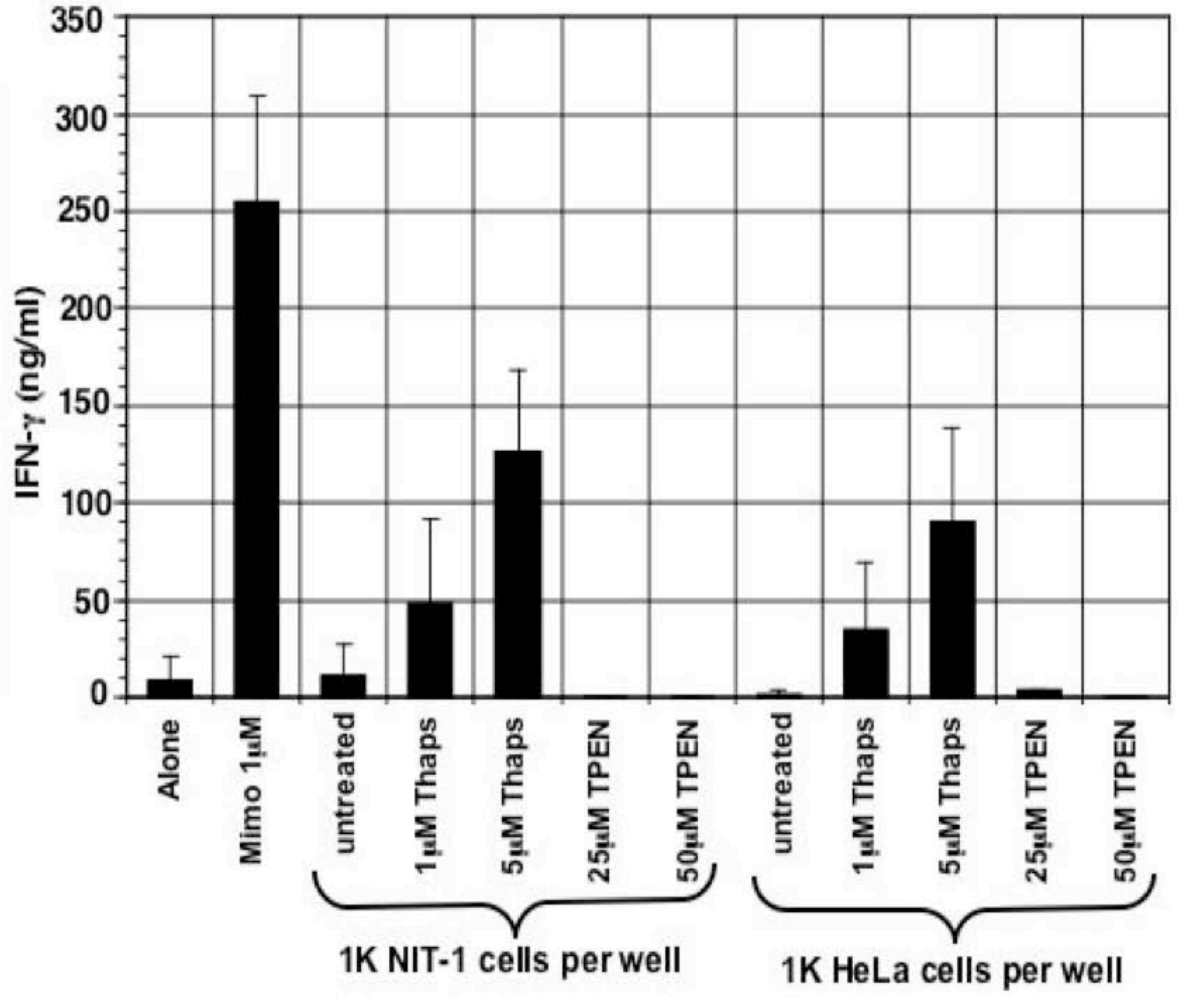

Figure 4.9 Upregulation of ER stress in NIT-1 and HeLa cells leads to an antigenic response by the BDC-2.5 T cell clone. *In Vitro* T Cell Assay: $2x10^4$ BDC-2.5 T cell clones and $5x10^5$ irradiated (3500 RAD) NOD splenocytes were incubated with one thousand (1K) NIT-1 cells treated with 0, 1, or 5μM thapsigargin or 25, or 50μM TPEN or 1K HeLa cells treated with 0, 1, or 5μM thapsigargin or 25, or 50μM TPEN. On day three, supernatants were collected for IFN-γ ELISA. The data are the average of three experiments.

Table 4.II Death Assay with NIT-1 cells treated with thapsigargin or TPEN.

	Live Cells	Apoptotic Cells	Membrane Damaged Cells	Membrane Damaged/Dead Cells
Control	58.3	0	21	18
1μM Thaps.	61	1	21.6	32
5μM Thaps.	59.6	1	17.6	23
10μM TPEN	25	22.5	29.75	10.2
50μM TPEN	30.75	16.5	20	33.6

NIT-1 cells were treated with 1 or 5μM thapsigargin for 1hr or 10 or 50μM TPEN for 4hr. Cells were then assessed by death assay using DNA intercalating dye mix. 100 cells were scored. Percentage for the morphological catergories, (1) viable cells with normal nuclei, (2) necrotic cells with normal nuclei, (3) apoptotic cells with intact membrane, and (4) apoptotic cells with damaged membrane, were calculated and reported.

Induction of ER stress in NIT-1 cells by thapsigargin treatment increases GRP78 expression (**Figure 4.8**), and positively correlates with an antigenic response from the BDC-2.5 T cell, as demonstrated by the increase in IFN-γ production compared to non-thapsigargin treated NIT-1 cells **(Figure 4.9)**. An immunoprecipitation reaction using BDC-2.5 TCR Tg serum proved to pull down increasing amounts of the GRP78, from NIT-1 cells treated with increasing concentrations of thapsigargin compared to untreated NIT-1 lysate (**Figure 4.8**). Since GRP78 is not unique to the islet beta cell, I asked whether the neo-antigenicity demonstrated by ER stress induction in the NIT-1 cells could also be demonstrated in non-beta cells by treating HeLa cells with thapsigargin and using them as a source of antigen in the BDC-2.5 T cell assay. Thapsigargin treated HeLa cells also elicited an antigenic response from BDC-2.5 as determined by elevated IFN-γ production (**Figure 4.9**). This response was not as marked as in the NIT-1 cells, however, the increase in overall GRP78 expression with thapsigargin treatment was not as robust as was demonstrated by the NIT-1 cells (**Figure 4.8**). This may be attributed to the HeLa cells being not of islet origin or because they are human in origin. In marked contrast, both non-thapsigargin treated NIT-1 and HeLa cells were unable to stimulate the BDC-2.5 T cell clone above background levels (**Figure 4.9**). Furthermore, to prove that this antigenicity induced by thapsigargin treatment was a result of ER stress and not apoptosis, NIT-1 cells were treated with TPEN, a zinc chelator capable of initiating apoptosis in cells (105). TPEN-induced apoptosis of NIT-1 cells did not increase antigenicity for the BDC-2.5 T cell clone (**Figure 4.9**), even though 10 and 50μM TPEN treatment resulted in 22.5% and 16.5% apoptotic cells, respectively. Interestingly, 1 and 5μM thapsigargin treatment only rendered 1% of NIT-1 cells apoptotic (**Table 4.II**) even though BDC-2.5-dependent IFN-γ synthesis increased 5 and 13-fold, respectively (**Figure 4.9**).

4.3 DISCUSSION

Autoimmune diseases arise as a result of a complex interplay of factors, but it is often considered that the failure of the immune system to recognize 'self' leads to a breakdown in tolerance. Exploiting this breakdown in self-tolerance, I have developed a novel technique, termed RISAT, to reconstitute NOD.*Rag*$^{-/-}$ mice with BDC-2.5 T cells and an open repertoire of NOD B cells. Upon multiple immunizations with βmem, a previously described antigenic protein prep for the BDC-2.5 T cell (62, 85), I was able to induce the production of antibodies that immunoprepicated the ER stress induced protein GRP78 from βmem as identified by MALDI-ToF analysis and Edman degradation. Additional bands detected by BDC-2.5 RISAT antibodies were not identified by MALDI-ToF or Edman degradation analysis. There are several reasons that I may not have been able to identify these proteins; these additional bands may be breakdown products or truncated proteins. Due to the techniques used, the failure to identify the bands may be the result of N-terminal blockage that is commonly the result of acetyl groups (106). Serum from non-immunized NOD.BDC-2.5 TCR Tg mice recognized GRP78 in the βmem preparation, confirming the RISAT-reconstitution results.

To determine if purified GRP78 protein could elicit a response *in vitro* from BDC-2.5 T cells, I obtained a commercially available recombinant form of the protein produced in *E. coli*. The *E. coli* produced recombinant protein failed to stimulate the production of IFN-γ by the BDC-2.5 T cell clone above background levels in an *in vitro* T cell assay. Since the RISAT data demonstrated that BDC-2.5 T cells could support T cell-dependent autoantibody production to

GRP78, I deduced that eukaryotic-dependent post-translational modification (PTM) of the protein might be necessary to confer antigenic recognition of GRP78 by BDC-2.5, an alteration to the protein that would be absent in a prokaryotic expression system. Support for this hypothesis comes from work in Rheumatoid Arthritis (RA). It is known that GRP78 is a target autoantigen in RA and both T and B cell autoreactivity has been demonstrated (83). Moreover, Blass et al., (83) reported that 63% of the 400 RA patients tested for T cell reactivity to GRP78 demonstrated specific T cell reactivity when challenged with biochemically purified GRP78 from mammalian cells. However, when these GRP78-specific T cells were screened against an *E. coli* derived GRP78 (the same commercially available source used in the BDC-2.5 T cell clone assays), there was no antigenic response, thus indicating that a PTM occurring in mammalian cells confers the antigenicity for this protein as an auto-antigenic target. These results may explain the lack of response from BDC-2.5 with the recombinant produced GRP78.

The results provide evidence that the BDC-2.5 T cell responds to GRP78 *in vivo*, but not to recombinant GRP78 *in vitro*. I therefore decided to test the hypothesis that induction of ER stress in a beta cell line would lead to the necessary modification of the target protein, GRP78, and confer antigenic recognition for the BDC-2.5 T cell. Since the BDC-2.5 T cells are not responsive towards NIT-1, a NOD-derived insulinoma cell line, I chose this line to test the hypothesis. NIT-1 cells treated with the ER stress inducer thapsigargin, demonstrated an increase in GRP78 expression, concomitant with increased IFN-γ secretion from BDC-2.5 T cells after stimulation with thapsigargin treated NIT-1 cells. Thapsigargin induced ER stress in HeLa cells also proved to elicit an IFN-γ response from the BDC-2.5 T cells, demonstrating that the ER stress induced upregulation of GRP78 confers antigenicity for the BDC-2.5, even in a

non-beta cell line. These results further support the hypothesis that a eukaryotic dependent modification of GRP78 is likely necessary for neo-antigenic recognition by BDC-2.5.

Although some of the identified autoantigens in T1D have proven to be unique to islet beta cells, other antigens have been determined to be widely expressed proteins, including for example the recently identified T cell antigen, dystrophia myotonica kinase (DMK). DMK is the antigen for the highly pathogenic CD8 T cell clones AI4 and its expression is not limited to islet beta cells (107). Interestingly, the recognition of widely expressed proteins leading to specific beta cell destruction point to the unique physiology of beta cells as a possible predisposing factor to auto-aggressive T cell reactivity, (96, 97, 108) that is highlighted by an increase in continuous ER stress. This further supports the idea that a unique post translational modification of the target protein GRP78 may confirm neoantigenicity. These results, demonstrating that ER stress induction of the beta cell adenoma NIT-1 leads to induced antigenic recognition by the BDC-2.5 T cell clone, point to the fascinating possibility that this physiological response in beta cell function during cellular stress may set in motion the activation of an autoreactive repertoire leading to beta cell destruction in individuals genetically predisposed for autoimmunity. Recent reports have highlighted that many of the hypothesized environmental triggers that induce T1D through beta cell apoptosis, also lead to ER stress (70, 73, 96). It is therefore tempting to speculate that at least for the diabetogenic T cell clone, BDC-2.5, that ER stress induction is necessary for antigenic recognition and adaptive immune effector function.

The increased antigenicity observed with thapsigargin treated NIT-1 cells was not due to apoptosis since treatment of NIT-1 cells with the apoptosis-inducing agent TPEN did not increase the response of BDC-2.5 T cells. These results support the hypothesis that cellular stresses, which disrupt normal ER function and activate the ER stress cascade, are a prerequisite

for antigenic-recognition by the BDC-2.5 T cell clone. Bypassing ER stress induction through direct apoptosis may not allow for a threshold level of GRP78 to be attained for efficient activation of BDC-2.5 T cells. Yet prolonged ER stress can eventually lead to apoptosis induction (71, 109, 110), which would facilitate the release of beta cell antigens that could be picked up by dendritic cells and ultimately presented to autoreactive T cells (96). Expanding on those observations further, I emphasize that the induction of ER stress in beta cells initiates the release of antigen(s) and generates an appropriate threshold level of antigen that activates an autoreactive repertoire of T cells, such as the BDC-2.5 T cell. Recently, Wang et al published that exposure of NIT-1 cells to streptozotocin (STZ) led to their upregulation of GRP78, as a protective mechanism against cell death (111). This effort to protect against beta cell death by inducing the ER stress protective program may be an inherent defense mechanism by the islet against environmental stressors. A predisposition toward autoimmunity, however, may result in activating the autoimmune system to the related stress proteins, such as GRP78, breaking self-tolerance, and initiating the self-destruction of the islet beta cells. Further support for this theory comes from Coxsackie viral-mediated diabetes induction models in the NOD.BDC-2.5 TCR Tg mice. Horwitz et al demonstrated that in the absence of direct viral-induced beta cell apoptosis, infection of NOD.BDC-2.5 TCR Tg mice with Coxsackie CB4 led to rapid development of diabetes by a non-specific bystander mechanism (112, 113). I would speculate that viral-induced ER stress would increase GRP78 expression over a threshold and activate NOD.BDC-2.5 TCR Tg T cells to promote beta cell destruction (114).

It has long been proposed that a number of environmental triggers (viral infection, drugs, and chemical inducers of oxidative stress) lead to the onset of T1D (114, 115), but a common link between these triggers has not been revealed. The data demonstrates that a key ER-

associated protein, GRP78, is an auto-antigenic target of the BDC-2.5 T cell clone in the RISAT-reconstituted immune system, and after chemically inducing ER stress in the non-antigenic NIT-1 cells. In both cases, an elevation of GRP78 expression resulted in a concomitant increase in antigenic recognition by BDC-2.5 T cell clone. In conclusion, these results strongly support ER stress as a mechanistic link for environmental triggers leading to the initiation of T cell-mediated beta cell recognition, destruction, and death in those individuals that are genetically predisposed to autoimmunity.

5.0 POSTTRANSLATIONAL MODIFICATION OF GLUCOSE REGULATED PROTEIN 78 IS RECOGNIZED BY THE BDC-2.5 TCR-TG SERA.

5.1 INTRODUCTION

'Conformational diseases' or 'folding diseases' are diseases caused by the misfolding of cellular protein, which leads to protein aggregates that initiate ER stress. It has become clear that ER stress plays a critical role in a number of diseases and efforts to elucidate the pathological contribution of ER stress are now under investigation. ER stress induction occurs under a specific program as follows, 1) induction of ER chaperones through the unfolded protein response (UPR), 2) transitional attenuation, 3) ER associated degradation (69), and 4) apoptosis (70).

IRE1 and PERK are held in the ER by GRP78 (Glucose regulated protein 78 kDa)/BIP (Immunoglobulin binding protein) and, upon ER stress induction, and released by GRP78 from the inner ER membrane to initiate the cascade of ER stress induced protein processing regulation. ATF6 is activated by a two-step cleavage by Site-1 protease (S1P) and Site-2 protease (S2P) and is the transcription factor necessary for the activation of new GRP78 m-RNA. IRE1 initiates XBP-1 to activate the transcription of target genes for molecular chaperones and accessory proteins such catalysts (68). The increased number of chaperones is able to re-process the proteins that were unfolded or misfolded.

Alterations in ER stress response have been shown in neurodegenerative diseases, bipolar disorder, diabetes both T1D and T2D, atherosclerosis, ischemia, heart disease, liver disease, kidney disease and inflammatory diseases. In T1D, inflammation plays a critical role in the disease progression and in some instances is associated with ER stress. It has been reported that during inflammation NO-induced apoptosis, mediated by ER stress and CHOP, has been demonstrated in beta cells (70, 81, 82) (69, 75). ER stress has also been reported to be important in autoimmunity. In rheumatoid arthritis, GRP78 a key regulatory protein in the ER stress pathway as a target autoantigen with both T and B cell autoreactivity, with reports of up to 63% of the RA patients demonstrating a specific T cell reactivity when challenged with biochemically purified GRP78 from mammalian cells (83).

Secretory cells, like islet beta cells and plasma cells, have a more developed ER in order to manage the continuous pressure of protein processing. This leads to continuous ER stress with upregulation of chaperones and accessory proteins in order to maintain ER homeostasis (80). In islet beta cells, disequilibrium in the ER leading to activation of ER stress occurs due to a number of different cellular events. There is increasing evidence that cytokines, reactive oxygen species (ROS), viral infection, and drug/toxin exposure results in activation of ER stress. ROS has been shown to be important in beta cell death in type 1 diabetes. Oyadomari et al demonstrated that the mouse insulinoma cell line, MIN6, produces nitric oxide (NO) in response to treatment with the cytokines, IL-1β, TNF-α, and IFN-γ. The production of NO correlates with the activation of ER stress, as was demonstrated by the upregulation of GRP78, IRE1a, and PERK (74).

Interestingly, caspase-12 mediated apoptosis has been theorized as a possible mechanism of antigen release for the mouse derived diabetogenic T cell clone BDC-2.5 T cell, a

diabetogenic $CD4^+$ Th1 T cell clone derived from a diabetic non-obese diabetic (NOD) female mouse (96). The BDC-2.5 recognizes islet beta cells and results in the induction of rapid diabetes in very young (<10 days-old) NOD recipients. The BDC-2.5 T cell clone however, fails to initiate diabetes in adult NOD.*scid* mice unless co-transferred with $CD8^+$ T cells from a diabetic NOD mouse. Both the RISAT technique and the BDC-2.5 TCR-transgenic (91) model demonstrate the BDC-2.5 T cell lends help to B cells to drive the production of IgG antibodies specific for GRP78. Additionally, the induction of GRP78 by the calcium flux caused by thapsigargin treatment leads to the BDC-2.5 T cell clone producing IFN-γ.

There is evidence that post-translational modifications (PTM) can lead to the formation of a neoantigen. PTM such as glycosylation, phosphorylation, and deamidation regulate the structure and function of proteins. Phosphorylation regulates enzyme activity, activates signal transduction and is involved in protein-protein interactions. Glycosylation is critical for protein stability, solubility, and secretion signals. Protein activity and interactions are also regulated by citrullination or deamidation.

Although these PTM have a functional cellular role, the protein changes that are caused by the PTM can elicit an immune response. Phosphorylation of lupus antigens is required for antibody recognition in the lupus mouse model, MRL/lpr (116). Viruses have coat viral proteins with N-linked glycosylation sites enabling these proteins to go undetected by the immune system, however, when the viral proteins are deglycosylated target epitopes become available for immune detection (117). There are cohorts of T cells that recognize citrullinated proteins in rheumatoid arthritis (RA) (118). Antibodies specific for anti-citrullinated proteins are also present in RA patients (119). These antibodies may arise from increased citrullinated proteins as a result of apoptotic events (33, 120).

There are several theories on the mechanism of PTM initiating an immune response (91). 1) The absence of PTM in the thymus during T cell maturation does not allow for T cells which recognize PTM epitopes to be negatively selected against. 2) PTMs affect antigen processing and thus presentation of antigenic epitopes. Cleavage sites necessary for antigenic epitope presentation can be blocked by PTM. For example, this occurs with myelin basic protein (MBP), an autoantigen in multiple sclerosis (31). Citrullination sites block cathepsin D from digesting MBP, thus altering how the protein is processed and presented by APCs to T cells (91, 121). 3) The binding of antigens in the MHC-II groove is affected by PTMs. Citrullinated RA antigens have a 20-100 times higher affinity to the RA-associated MHC-II molecules (DRB1*0404, *0401 and *0101) compared to unmodified antigens (122). 4) There is an association of PTM with cellular stress and apoptosis. Utz et al demonstrated that proteins phosphorylated during stress inducing apoptosis are autoantibody targets in lupus (123). PTM antigens are concentrated in blebs from apoptotic cells (124, 125) and may enable the levels of antigen to exceed the antigen threshold necessary for triggering the autoreactive immune response. Hence, I explored the role of several different PTM to determine if they play a role in the development of GRP78 becoming antigenic for the BDC-2.5 T cell clone.

In order to further understand how the global expressed GRP78 may lead to an organ-specific destruction, I attempted to purify stress-induced GRP78. Through immunoblot analysis, I was able to determine that GRP78 has sites of o-linked glycosylation and citrullination. I also confirmed that GRP78 is phosphorylated on threonine and serine residues in NIT-1 cells. Additionally, I found the BDC-2.5 TCR-Tg antibodies recognize a subset of GRP78 which is modified with phosphoserine. The data presented further elucidates how GRP78 develops into the neoantigen for the BDC-2.5 T cell clone.

5.2 RESULTS

5.2.1 Individual peptides from GRP78 peptide library fail to stimulate the BDC-2.5 T cell clone.

15mer peptides were synthesized for the GRP78 (**Table 5.I**). The 161-15mers were pooled into 26 sets of twelve to thirteen peptides (**Table 5.II**). Pools were tested for antigenicity against the BDC-2.5 T cell clone. Pools IX and XVI resulted in stimulation of IFN-γ production, however, when I looked at the common (p35) and surrounding peptides (p32 to p37), they failed to stimulate the BDC-2.5 T cell clone.

Table 5.I Peptide Library for GRP78.

ID #	Sequence	ID #	Sequence	ID #	Sequence
1	MMKFTVVAAALLLLGA	55	GEKNILVFDLGGGTFD	109	VMTKLIPRNTVVPTKK
2	TVVAAALLLLGAVRAE	56	ILVFDLGGGTFDVSLL	110	LIPRNTVVPTKKSQIF
3	AALLLLGAVRAEEEDK	57	DLGGGTFDVSLLTIDN	111	NTVVPTKKSQIFSTAS
4	LLGAVRAEEEDKKEDV	58	GTFDVSLLTIDNGVFE	112	PTKKSQIFSTASDNQP
5	VRAEEEDKKEDVGTVV	59	VSLLTIDNGVFEVVAT	113	SQIFSTASDNQPTVTI
6	EEDKKEDVGTVVGIDL	60	TIDNGVFEVVATNGDT	114	STASDNQPTVTIKVYE
7	KEDVGTVVGIDLGTTY	61	GVFEVVATNGDTHLGG	115	DNQPTVTIKVYEGERP
8	GTVVGIDLGTTYSCVG	62	VVATNGDTHLGGEDFD	116	TVTIKVYEGERPLTKD
9	GIDLGTTYSCVGVFKN	63	NGDTHLGGEDFDQRVM	117	KVYEGERPLTKDNHLL
10	GTTYSCVGVFKNGRVE	64	HLGGEDFDQRVMEHFI	118	GERPLTKDNHLLGTFD
11	SCVGVFKNGRVEIIAN	65	EDFDQRVMEHFIKLYK	119	LTKDNHLLGTFDLTGI
12	VFKNGRVEIIANDGGN	66	QRVMEHFIKLYKKKTG	120	NHLLGTFDLTGIPPAP
13	GRVEIIANDGGNRITP	67	EHFIKLYKKKTGKDVR	121	GTFDLTGIPPAPRGVP
14	IIANDGGNRITPSYVA	68	KLYKKKTGKDVRKDNR	122	LTGIPPAPRGVPQIEV
15	DGGNRITPSYVAFTPE	69	KKTGKDVRKDNRAVQK	123	PPAPRGVPQIEVTFEI
16	RITPSYVAFTPEGERL	70	KDVRKDNRAVQKLRRE	124	RGVPQIEVTFEIDVNG
17	SYVAFTPEGERLIGDA	71	KDNRAVQKLRREVEKA	125	QIEVTFEIDVNGILRV
18	FTPEGERLIGDAAKNQ	72	AVQKLRREVEKAKRAL	126	TFEIDVNGILRVTAED
19	GERLIGDAAKNQLTSN	73	LRREVEKAKRALSSQH	127	DVNGILRVTAEDKGTG
20	IGDAAKNQLTSNPENT	74	VEKAKRALSSQHQARI	128	ILRVTAEDKGTGNKNK
21	AKNQLTSNPENTVFDA	75	KRALSSQHQARIEIES	129	TAEDKGTGNKNKITIT
22	LTSNPENTVFDAKRLI	76	SSQHQARIEIESFFEG	130	KGTGNKNKITITNDQN
23	PENTVFDAKRLIGRTW	77	QARIEIESFFEGEDFS	131	NKNKITITNDQNRLTP
24	VFDAKRLIGRTWNDPS	78	EIESFFEGEDFSETLT	132	ITITNDQNRLTPEEIE
25	KRLIGRTWNDPSVQQK	79	FFEGEDFSETLTRAKF	133	NDQNRLTPEEIERMVN
26	GRTWNDPSVQQKIKFL	80	EDFSETLTRAKFEELN	134	RLTPEEIERMVNDAEK
27	NDPSVQQKIKFLPFKV	81	ETLTRAKFEELNMDLF	135	EEIERMVNDAEKFAEE
28	VQQKIKFLPFKVVEKK	82	RAKFEELNMDLFRSTM	136	RMVNDAEKFAEEDKKL
29	IKFLPFKVVEKKTKPY	83	EELNMDLFRSTMKPVQ	137	DAEKFAEEDKKLKERI
30	PFKVVEKKTKPYIQVD	84	MDLFRSTMKPVQKVLE	138	FAEEDKKLKERIDTRN
31	VEKKTKPYIQVDIGGG	85	RSTMKPVQKVLEDSDL	139	DKKLKERIDTRNELES
32	TKPYIQVDIGGGGTKT	86	KPVQKVLEDSDLKKSD	140	KERIDTRNELESYAYS
33	IQVDIGGGQTKTFAPE	87	KVLEDSDLKKSDIDEI	141	DTRNELESYAYSLKNQ
34	IGGGQTKTFAPEEISA	88	DSDLKKSDIDEIVLVG	142	ELESYAYSLKNQIGDK
35	**QTKTFAPEEISAMVLT**	89	KKSDIDEIVLVGGSTR	143	YAYSLKNQIGDKEKLG
36	FAPEEISAMVLTKMKE	90	IDEIVLVGGSTRIPKI	144	LKNQIGDKEKLGGKLS
37	EISAMVLTKMKETAEA	91	VLVGGSTRIPKIQQLV	145	IGDKEKLGGKLSSEDK
38	MVLTKMKETAEAYLGK	92	GSTRIPKIQQLVKEFF	146	EKLGGKLSSEDKETME
39	KMKETAEAYLGKKVTH	93	IPKIQQLVKEFFNGKE	147	GKLSSEDKETMEKAVE
40	TAEAYLGKKVTHAVVT	94	QQLVKEFFNGKEPSRG	148	SEDKETMEKAVEEKIE
41	YLGKKVTHAVVTVPAY	95	KEFFNGKEPSRGINPD	149	ETMEKAVEEKIEWLES
42	KVTHAVVTVPAYFNDA	96	NGKEPSRGINPDEAVA	150	KAVEEKIEWLESHQDA
43	AVVTVPAYFNDAQRQA	97	PSRGINPDEAVAYGAA	151	EKIEWLESHQDADIED
44	VPAYFNDAQRQATKDA	98	INPDEAVAYGAAVQAG	152	WLESHQDADIEDFKAK
45	FNDAQRQATKDAGTIA	99	EAVAYGAAVQAGVLSG	153	HQDADIEDFKAKKKEL
46	QRQATKDAGTIAGLNV	100	YGAAVQAGVLSGDQDT	154	DIEDFKAKKKELEEIV
47	TKDAGTIAGLNVMRII	101	VQAGVLSGDQDTGDLV	155	FKAKKKELEEIVQPII
48	GTIAGLNVMRIINEPT	102	VLSGDQDTGDLVLLDV	156	KKELEEIVQPIISKLY
49	GLNVMRIINEPTAAAI	103	DQDTGDLVLLDVCPLT	157	EEIVQPIISKLYGSGG
50	MRIINEPTAAAIAYGL	104	GDLVLLDVCPLTLGIE	158	QPIISKLYGSGGPPPT
51	NEPTAAAIAYGLDKRE	105	LLDVCPLTLGIETVGG	159	SKLYGSGGPPPTGEED
52	AAAIAYGLDKREGEKN	106	CPLTLGIETVGGVMTK	160	GSGGPPPTGEEDTSEK
53	AYGLDKREGEKNILVF	107	LGIETVGGVMTKLIPR	161	PPPTGEEDTSEKDEL
54	DKREGEKNILVFDLGG	108	TVGGVMTKLIPRNTV		

Table 5.II Peptide Pool of GRP78 Peptide Library.

	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
XIV	1	2	3	4	5	6	7	8	9	10	11	12	13
XV	14	15	16	17	18	19	20	21	22	23	24	25	26
XVI	27	28	29	30	31	32	33	34	**35**	36	37	38	39
XVII	40	41	42	43	44	45	46	47	48	49	50	51	52
XVIII	53	54	55	56	57	58	59	60	61	62	63	64	65
XIX	66	67	68	69	70	71	72	73	74	75	76	77	78
XX	79	80	81	82	83	84	85	86	87	88	89	90	91
XXI	92	93	93	94	95	96	97	98	99	100	101	102	103
XXII	104	105	106	107	108	109	110	111	112	113	114	115	116
XXIII	117	118	119	120	121	122	123	124	125	126	127	128	129
XXIV	130	131	132	133	134	135	136	137	138	139	140	141	142
XV	143	144	145	146	147	148	149	150	151	152	153	154	155
XVI	156	157	158	159	160	161							

5.2.2 Freshly ATP 8-AH purified GRP78 is antigenic to the BDC-2.5 T cell clone.

GRP78 was purified by taking advantages of its ATP binding domain. Freshly thapsigargin-treated NIT-1 cells were lysed and subjected to an ATP column. The resultant elution was silver stained, immunoblotted, and put into a BDC-2.5 T cell assay. Purified GRP78 from Thapsigargin treated NIT-1 cells resulted in the stimulation of the BDC-2.5 T cell (**Figure 5.1**). Due to preparation-to-preparation variability in T cell assays, the same purified sample was utilized for a repeat assay (126). After the preparation underwent a freeze-thaw, it was no longer antigenic for the BDC-2.5 T cell clone (data not shown). I performed another set of biochemical assays, i.e. silver stains and immunoblots, to correlate with the results of the BDC-2.5 T cell assay. Serendipitously, both silver stains (**Figure 5.2**) and immunoblots (**Figure 5.3**) demonstrated a loss of the high MW band that was present in the antigenic elution that correlated with the loss of IFN-γ production by the BDC-2.5 T cell clone.

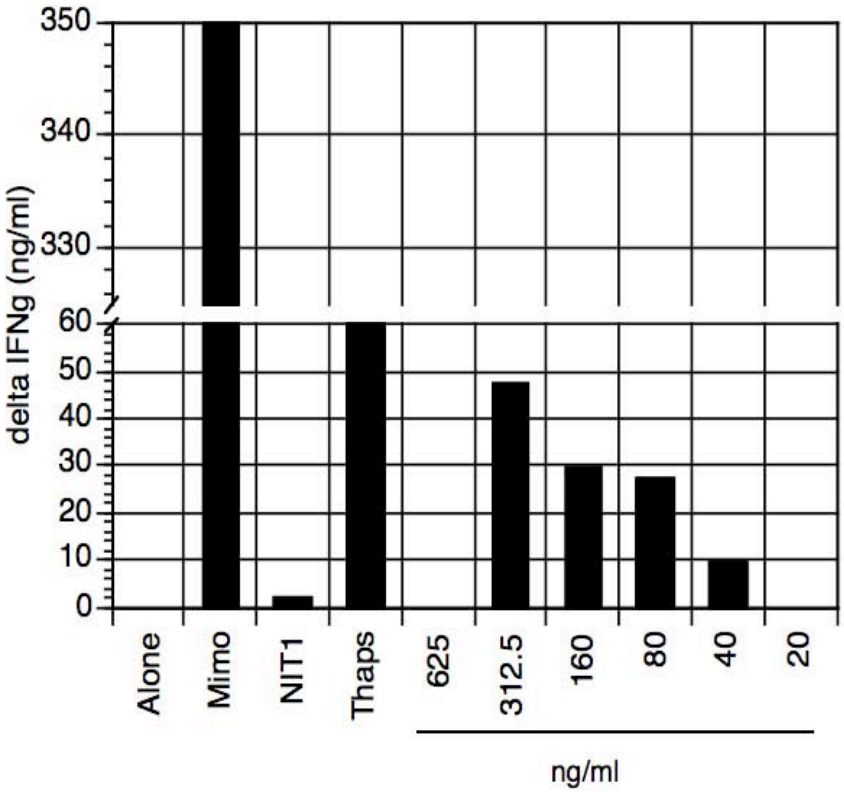

Figure 5.1 BDC-2.5 T cell assay of purified GRP78 from NIT-1 thapsigargin treated lysates. NIT-1 cells were treated with thapsigargin for 1 hour at 5μM. Cells were immediately lysed and purified using ATP 8-AH sepharose purification columns. Solid bars represent purified protein that was freshly assessed for antigenic by BDC-2.5 T cell assay, silver stain, and immunoblot. ELISA for IFN-γ was preformed to detect activation of the BDC-2.5 T cell clone. Mimo, positive control; Thaps, 5uM thapsigargin treatment of NIT-1 cells.

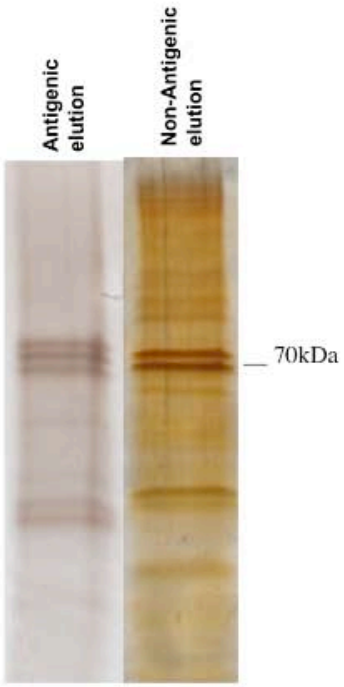

Figure 5.2 Silver Stain of purified GRP78 from NIT-1 thapsigargin treated lysates. NIT-1 cells were treated with thapsigargin for 1 hour at 5μM. Cells were immediately lysed and purified using ATP 8-AH sepharose purification columns. Lane A was freshly assessed for antigenic by BDC-2.5 T cell assay, silver stain, and immunoblot. Lane B underwent a freeze-thaw before assessment of antigenic by BDC-2.5 T cell assay, silver stain, and immunoblot.

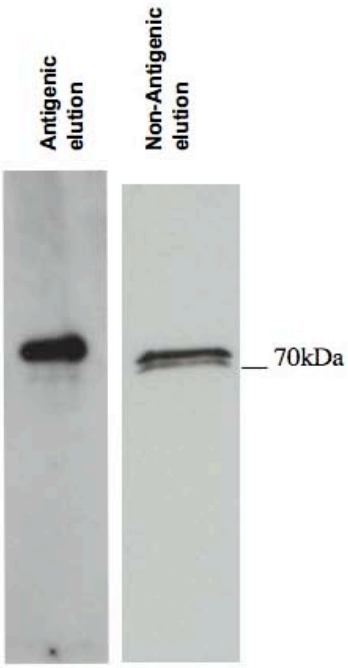

Figure 5.3 Immunoblot of purified GRP78 from NIT-1 thapsigargin treated lysates. NIT-1 cells were treated with thapsigargin for 1 hour at 5μM. Cells were immediately lysed and purified using ATP 8-AH sepharose purification columns. Lane A was freshly assessed for antigenicity by BDC-2.5 T cell assay, silver stain, and immunoblot. Membrane was probed with Santa Cruz (C-20) GRP78 antibody. Lane B underwent a freeze-thaw before assessment of antigenicity by BDC-2.5 T cell assay, silver stain, and immunoblot. Membrane was probed with Santa Cruz (N-20) GRP78 antibody.

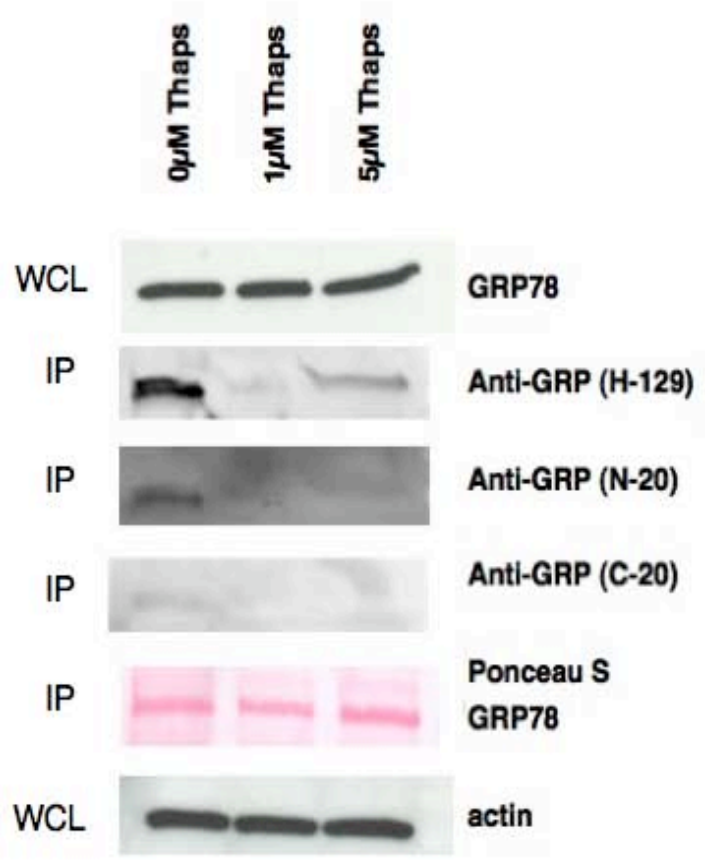

Figure 5.4 Thapsigargin alters antibodies ability to detect GRP78 pulled down by BDC-2.5 TCR-Tg. HeLa cells were treated with thapsigargin, 1 hour at either 0, 1, or 5 μM. Cells were lysed. Lysates were immunoprepicated with BDC-2.5 TCR-Tg serum overnight. The pulled down (IP) proteins were separated along side whole cell lysate (WCL). Pulled down proteins were probed with Santa Cruz anti-GRP78 (N-20), (C-20), (H-129). Whole cell lysates were probed with Santa Cruz anti-GRP78 (N-20) and actin. Membranes were stained with ponceau S before blocking and probing.

5.2.3 Thapsigargin treatment alters antibody recognition to GRP78 in HeLa cells.

Due to the poor cross-reactivity of the Stressgen anti-hamster GRP78 to human GRP78, I used anti-GRP78 antibodies from Santa Cruz to detect GRP78 in the human HeLa cell line. I performed immunoprecipitations on thapsigargin treated HeLa with BDC-2.5 TCR-Tg serum. The BDC-2.5 TCR-Tg serum successfully pulled down a 70 kDa protein corresponding to GRP78 as shown with ponceau S stain (**Figure 5.4**). Immunoblots of whole cell lysates showed that GRP78 was present in the lysate, however, attempts to probe the pulled down protein with three different Santa Cruz anti-GRP78 antibodies only yielded substantial detection of GRP78 in the untreated condition. The various antibodies recognize varying degrees of the GRP78 from thapsigargin treated NIT-1 cells, however, none of the antibodies recognize all of the GRP78 which is detected via ponceau S stain.

5.2.4 N-linked and O-linked glycosylation does not play a role in induction of antigenicity.

The inability to elicit a strong IFN-γ response with the overlapping peptides from GRP78 combined with the antibody data, led us to speculate that a post translational modification of the protein was occurring that was responsible for the antigenic recognition by BDC-2.5. To assess the role of PTM via glycosylation, I treated NIT-1 cells with tunicamycin, an inhibitor of N-linked glycosylation. The results demonstrated that N-linked glycosylation was not responsible for induction of antigenicity, since there was no change in IFN-γ production (data not shown) when NIT-1 cells were treated with thapsigargin and tunicamycin and then co-cultured in a BDC-2.5 T cell assay. In **Figure 5.5**, NIT-1 cells untreated and treated have numerous proteins that have sites of O-linked glycosylation; therefore I removed the O-linked glycosylation with

sodium hydroxide (**Figure 5.5**) and used the modified GRP78 protein in a T cell assay with BDC-2.5. I was not able to correlate the removal of O-linked glycosylation with antigenicity from the BDC-2.5 T cell clone.

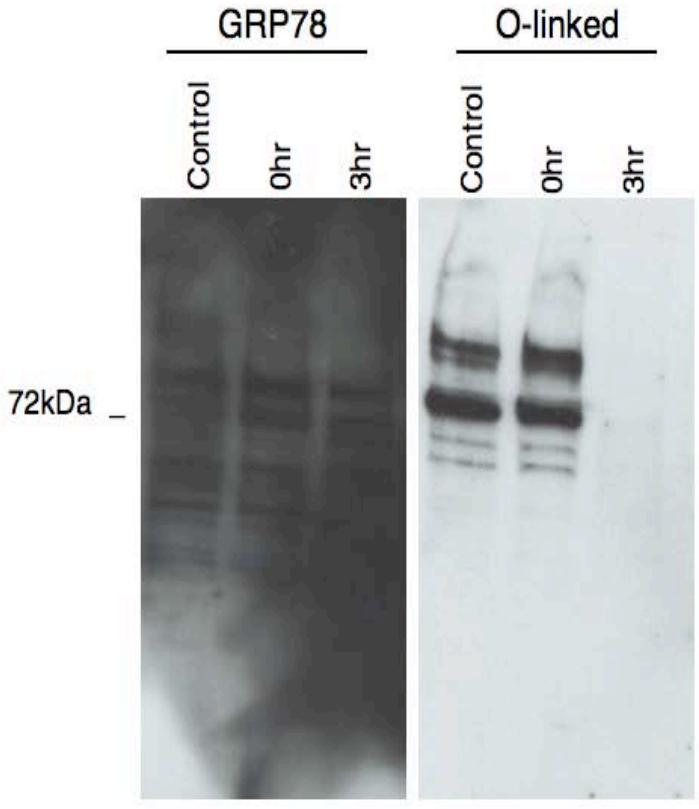

Figure 5.5 Removal of O-linked glycosylation sites on GRP78. NIT-1 cells were treated with thapsigargin, 1 hour at 5μM. Cells were lysed. Lysates were incubated with sodium hydroxide to deglycosylate proteins. Acetic acid was added to terminate the reaction either immediately after the addition of sodium hydroxide or after 3 hr incubation. Lysates were separated. Membranes were probed with either Stressgen anti-GRP78 or anti-O-linked glycosylation antibody.

5.2.5 GRP78 is citrullinated in NIT-1 cells.

With the current literature demonstrating the role of citrullination of proteins leading to increased self-recognition of proteins in RA, I explored this PTM as a possible mechanism of neoantigenicity. Citrullinated GRP78 was detected in NIT-1 lysates, however, there was no correlation between untreated and 5mM thapsigargin (data not shown). Additionally, the BDC-2.5 TCR-Tg serum did not remove all the citrullinated GRP78 as there was still detection of citrullinated GRP78 in the supernatant (**Figure 5.6A**). There was a dramatic change in the molecular weight of purified GRP78 following treatment with PAD, the enzyme that catalyzes the citrullination reaction (**Figure 5.6B**). This did not correlate with changes in the antigenic response by the BDC-2.5 T cell clone (**Figure 5.6C**).

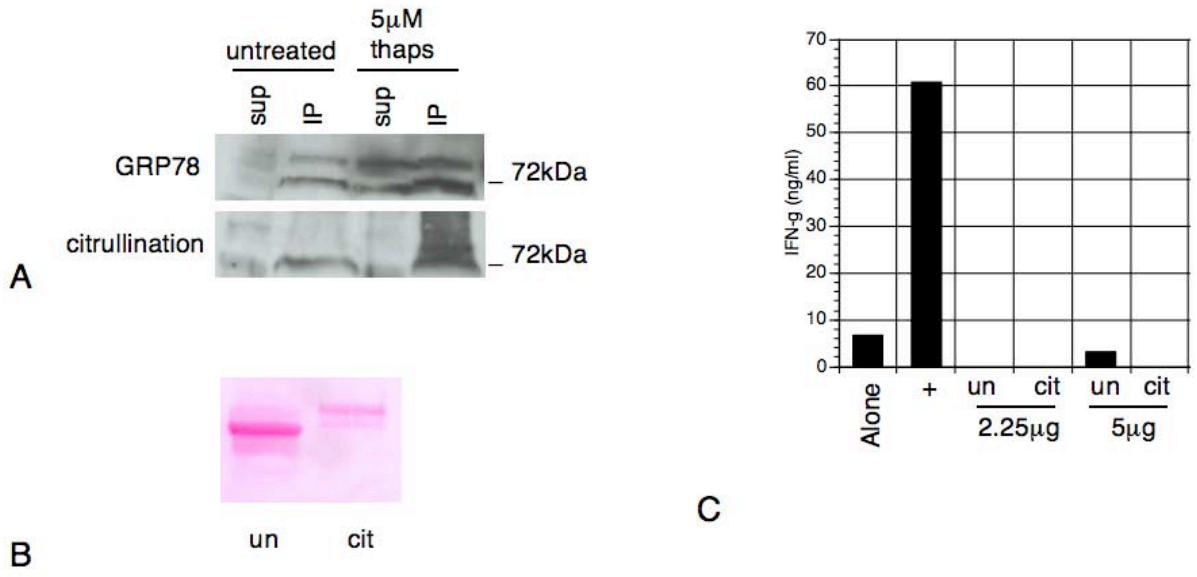

Figure 5.6 GRP78 is citrullinated. (A) NIT-1 cells were treated with thapsigargin, 1 hour at 5μM. Cells were lysed. Lysates were immunoprepicated with BDC-2.5 TCR-Tg serum overnight. The supernatants (sup) and pulled down (IP) proteins were separated. GRP78 was detected using anti-GRP78 from Stressgen. Citrullination was detected by use of Upstate Citrullination Detection Kit according to manufacture's instructions. (B) Citrullinated GRP78 was separated. Membranes were stained with ponceau S to determine if there was a change in the molecular weight after PAD treatment. (un) untreated. (cit) treated with PAD. (C) Citrullinated GRP78 was assayed with BDC-2.5 T cell clone. Commercial available purified GRP78 was treated with PAD to induce citrullination. Mimotope was used as a positive control.

5.2.6 Phosphorylation of serine residues.

Through immunoblots, I determined the phosphorylation profile of GRP78 in NIT-1 and HeLa (**Figure 5.7A**). GRP78 in both NIT-1 and HeLa contain phospho-tyrosine, threonine, and serine. I immunoprecipitated fresh lysates with the BDC-2.5 TCR Tg serum to determine if the serum antibodies were specific for a phosphorylation modification. Probing back with a phospho-

serine specific antibody, it was determined that the BDC-2.5 TCR Tg antibody recognizes a phospho-serine site(s) on GRP78 (**Figure 5.7B**).

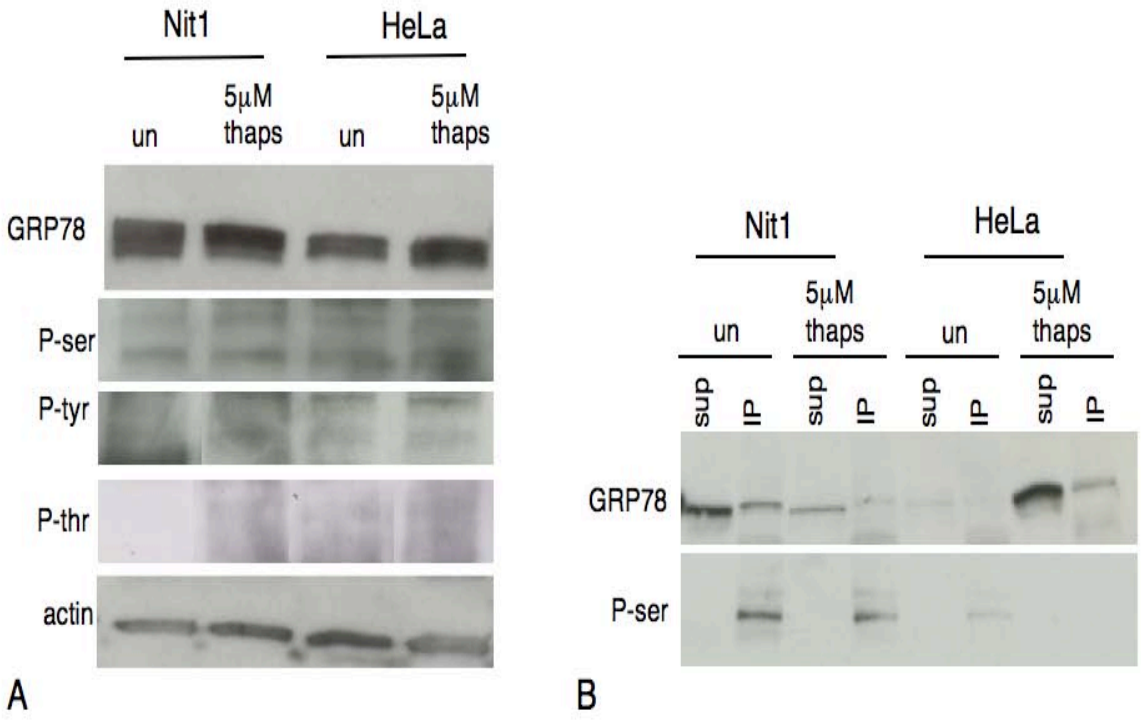

Figure 5.7 Phosphoserine is specific to GRP78 pulled down by BDC-2.5 TCR-Tg. NIT-1 and HeLa cells were treated with thapsigargin, 1 hour at 5μM. Cells were lysed. Untreated (un) and treated lysates were immunoprepicated with BDC-2.5 TCR-Tg serum overnight. (A) Whole cell lysates, (B) supernatants (sup) and pulled down (IP) proteins were separated. GRP78 was detected using anti-GRP78 (Santa Cruz N-20). Phosphoserine (P-ser), phosphotyrosine (P-tyr) and phosphothreonine (P-thr) were detected with Sigma phospho-specific antibodies. Actin was probed as a loading control for whole cell lysates.

5.3 DISCUSSION

Although, the BDC-2.5 T cell clone helps B cells to produce antibody to GRP78, recombinant expressed GRP78 does not elicit a response by the BDC-2.5 T cell clone (unpublished data, Schreiner et al). Freshly purified GRP78 from thapsigargin treated NIT-1 cells does lead to IFN-γ production by the BDC-2.5 T cell clone, however, after a freeze-thaw the purified protein losses antigenicity. Leustek et al determined that the *in vivo* and *in vitro* phosphorylation pattern of GRP78 is different (127). In vivo GRP78 has sites of phosphothreonine and phosphoserine, however, upon certain purification methods or *in vitro*, sites of phosphoserine are lost. There is evidence to support that the BDC-2.5 TCR-Tg antibodies clearly recognize a phosphoserine as phosphoserine GRP78 is void in the supernatant.

It cannot be ruled out that multiple PTM are necessary for GRP78 becoming antigenic. It has been proposed that citrullination leads to neoantigens because previously hidden epitopes become available. Due to the enzymatic conversion of arginine to citrulline, there is a change in charge from positive to neutral. This change leads to alterations in intramolecular protein binding causing the protein to unfold or fold differently.

Through immunoblot analysis I confirmed previous studies, which demonstrate biochemically purified GRP78 is modified to contain phosphoserine (127-129). It appears phosphoserine plays a critical role in the BDC-2.5 TCR-Tg serum being able to recognize GRP78 as the GRP78 remaining in the supernatant after clearing lysates with BDC-2.5 TCR-Tg sera is void of detectable phosphoserine. The limitations of immunoblot does not allow us to

identify how many or what serines are phosphorylated. Additionally, I was not able to determine if there are changes to phosphorylation sites with thapsigargin treatment. Identification of specific posttransitional modifications for individual amino acids can be achieved by various mass spectrometry techniques.

The BDC-2.5 TCR-Tg sera has poor affinity for denatured GRP78, evidence that it recognizes a conformational epitope. Conformational epitopes are against 15-22 protein residues that are scattered over two or more discontinuous polypeptide segments (130, 131). In infectious diseases, i.e. equine infectious anemia virus (EIAV) and parvovirus, the initial antibody response is to linear epitopes, however, over time there is a shift and selection for antibodies that recognize conformational epitopes indicative of a mature immune response (132, 133). By the time a patient has clinical symptoms of diabetes, the immune response has been on going with the majority of beta cells already destroyed. In some cases the immune response may have been initiated months or years prior (3). Currently it is believed that in autoimmunity the majority of autoantibodies are to conformational epitope (131), though, it is not clear if autoimmune diseases go through the same linear to conformational maturation of antibody production as infectious diseases, due to the inability to properly monitor antibody production in autoimmune individuals.

Epitope mapping using a GRP78 peptide library modified to contain phosphoserine is a way to identify whether the B cell epitope for the BDC-2.5 TCR-Tg sera is against a linear or conformational epitope. The BDC-2.5 antibody may sufficiently bind a modified peptide to identify a portion of the B cell epitope. Phage library screening has been successful in identifying conformational epitopes (130).

It is unclear if the T cell receptor sees the same epitope as the B cell receptor. The GRP78, which the B cell internalizes, processes, and presents to the BDC-2.5 T cell, contains

phosphoserines. The binding surface that is antigenic to the BDC-2.5 may contain different sites of modification, such as phosphothreonine, citrulline, o-linked glycosylation, all of which I demonstrate occur in NIT-1 treated with thapsigargin, than that which are required for B cell recognition. Presented within the antigen-binding groove of MHC molecules, T cell epitopes are short linear peptides (131). A GRP78 peptide library that has been modified to contain PTMs should yield the identification of the BDC-2.5 T cell epitope along with the necessary PTM for antigenicity.

Even though the window of disease transfer with the BDC-2.5 is early in mouse postnatal development, it remains to be determined what role the BDC-2.5 T cell and its cognate antigen, GRP78, plays in the initiation and progression of T1D. Furthermore, GRP78 and ER stress may be shown to the common link between the many potential environmental triggers proposed to initiate T1D. Additionally, these findings may lead to potential therapies to eliminate the immune response to GRP78 during events of ER stress. Other autoimmune diseases and ER stress disorders may benefit from application of potential therapies.

6.0 CONCLUSIONS

6.1 PROPOSED MODEL OF GRP78 AND BDC-2.5 INTERACTION

As the proposed model in **Figure 6.1** shows, individuals with a genetic predisposition to developing diabetes have poor central and peripheral regulation of lymphocytes allowing autoreactive T and B cells in circulation. Islet beta cells, as stated, are incredibly sensitive to ER perturbation and their continuous need to produce, package and secrete insulin upon glucose stimulated signaling provides a nonstop strain of protein production that is poised to ER stress. In combination with environmental triggers such as viral infection, chemical toxins, or food exposure, ER stress is further exacerbated leading to increased GRP78 production. Changes in calcium levels in the ER lead to the release of GRP78 and the initiation of the UPR and ER dysregulation. Increased levels of GRP78 are produced in an effort to control protein packaging and processing problems that occur during exposure to viruses, chemicals, and foods that inducescellular stresses such as oxidative stress. The upregulation and PTM of GRP78 alters this protein enough for the immune system of genetically predisposed individuals to see this self-protein as foreign. Modified GRP78 is engulfed by APCs that process and present peptide fragments to circulating T cells. The BDC-2.5 T cell becomes activated upon being presented its antigen peptide. The activated BDC-2.5 T cell proliferates and produces IFN-γ leading to the recruitment of accessory cells to the islets, which initiates islet-beta cell destruction. Following

presentation of its antigenic epitope, the activated T cell will lend help to the presenting B cell. The B cell matures into a plasma cell and secretes IgG antibodies. The immune system will utilize these antibodies in antibody dependent cell cytotoxicity.

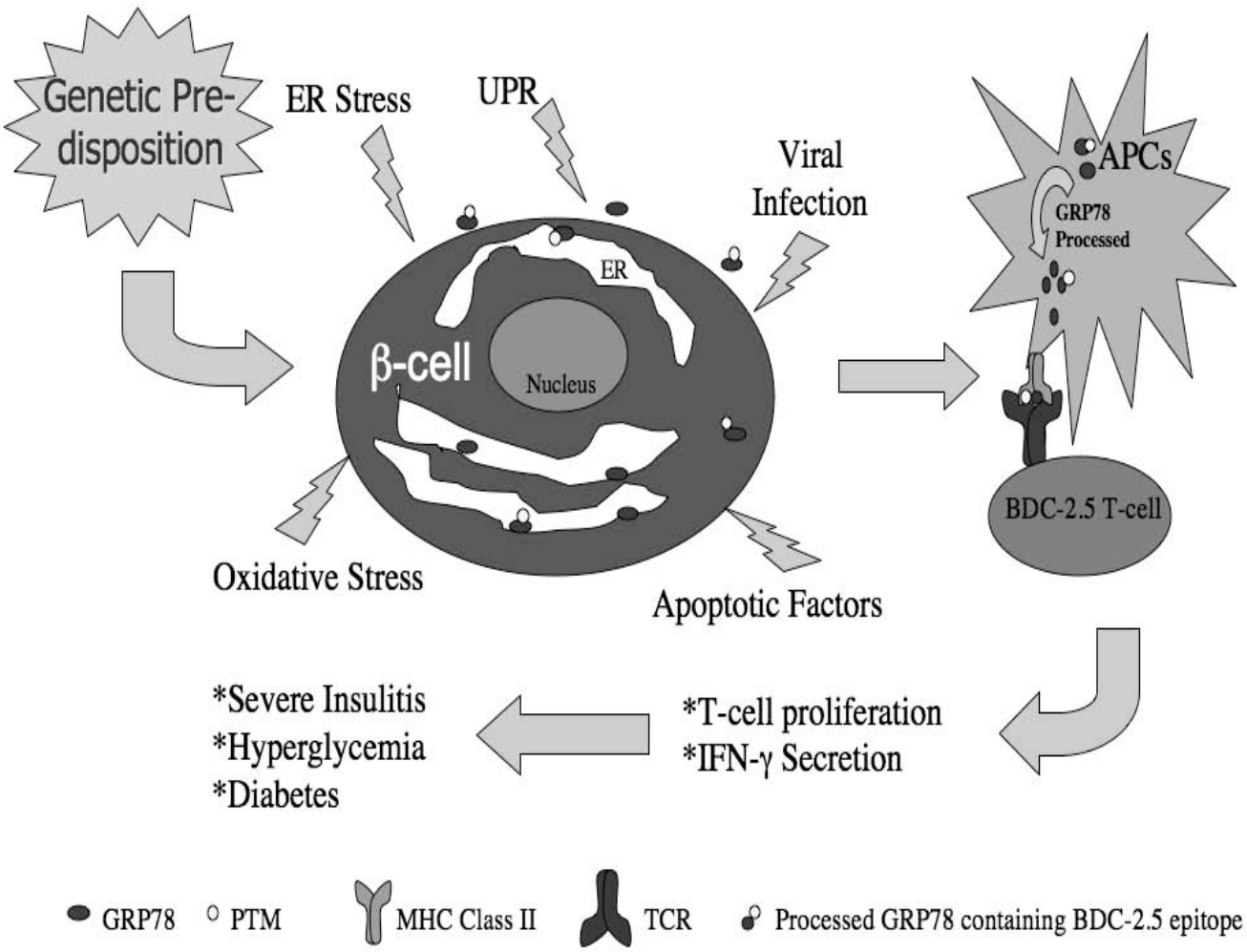

Figure 6.1 Proposed Model of GRP78 and BDC-2.5 Interaction.

6.2 EVIDENCE TO SUPPORT THE MODEL OF INTERACTION

Identification of a common link between environmental triggers, i.e. viral infection, chemical and food exposure, would provide tremendous insight to the pathogenesis of T1D. The presence of BDC-2.5 T cell's antigen has been previously demonstrated in a number of environmental triggers with the activation of the BDC-2.5 T cell clone. There is also increasing evidence for ER stress, more specially GRP78, playing a role in environmental triggers. The cell attempts to protect itself from apoptosis initiated by environmental triggers leads to the upregulation of neoantigens, such as GRP78.

Although GRP78 is expressed in numerous cell types, beta cells have inherently elevated levels of GRP78 as a result of their role as secretory cells constantly undergoing ER stress. Iwawaki et al demonstrated that the pancreas is the first organ with massive ER stress using the "ER stress-activated indicator" (ERAI) mouse model (68). The onset of massive pancreatic ER stress coincided with the reported window in which the BDC-2.5 T cell clone can transfer diabetes in the NOD and NOD.*scid* mice. After this window, initiation of cellular damage with a cotransfer of diabetic $CD8^+$ T cells must accompany the BDC-2.5 T cells to lead to insulitis and hyperglycemia (85).

Streptozotocin (STZ), a drug commonly used in TID research to abolish the beta cell mass, allows for synchronized onset of diabetes and may mimic the environmental trigger of chemical exposure. The BDC-2.5 T cells activate and drain to the pancreatic lymph node within 66 hours of being adoptively transferred into STZ treated NOD mice (96). Exposure of NIT-1

cells to STZ leads to upregulation of GRP78 as a protective mechanism against cell death (111). Caspase-12 mediated death is necessary for the release of the BDC-2.5 T cell antigen as blocking of caspase-mediated death by using either benzyloxylcarbonyl-V-A-D-*O*-methyl fluoromethyl ketone (ZVAD) or caspase-12 knockout mice resulted in the BDC-2.5 T cell not becoming activated (96). ER stress-apoptosis mediated through caspase-12 may be a mechanism that allows for the release and presentation of modified GRP78.

The model lends rationale to my hypothesis that dysregulation in beta cell function as a result of various environmental triggers leads to increased ER stress, upregulation and PTM of GRP78, and subsequent aberrant recognition of GRP78 by the immune system that initiates T1D. Evidence that supports this comes from studies of viral infections, specifically coxsackievirus B4 (CVB4), which have shown homology exists between viral and self-protein sequences, CVB4-2C and GAD65 for instance (16, 17). Furthermore, Horwitz et al demonstrates that the BDC-2.5 TCR-Tg mice develop extensive insulitis and overt diabetes when infected with CVB4 (112). Although, the role of GRP78 has not been shown with CVB4 viral strain, GRP78 has been identified as a surface coreceptor in other coxsackeivirus (A9) infections (134).

The mechanism of food exposure as an environmental trigger of T1D has been associated with BDC-2.5 T cell activation. In studies performed by Turley et al, mice were fed dextran sodium sulfate (DSS) to mimic large intestine injury that occurs during inflammation caused by food exposure (135). Following treatment with DSS, NOD mice were adoptively transferred with purified BDC-2.5/NOD T cells. Even though the cellular damage occurred in the gut, activated BDC-2.5 T cells were located in the pancreatic lymph node. Additionally, treatment of BDC-2.5/NOD animals with DSS resulted in a higher frequency of insulitis compared to control mice. DSS is commonly used in colitis studies for diseases such Crohn's, Celiac, and

inflammatory bowel disease (IBD). Mice treated with DSS have increased GRP78 expression in their colons (136). Examination of human colons from individuals with colitis and Crohn's disease also demonstrates evaluated levels of GRP78 (137).

The literature outlined above illustrates the strong parallels between the BDC-2.5 antigen, GRP78, ER stress and, T1D environmental triggers. In order to confirm that ER stress, more specially GRP78, is the common link between T1D environmental triggers, future experiments should use the NOD mice and tissue from diabetic humans to dissect ER stress and GRP78's role in the development of T1D.

6.3 FUTURE DIRECTIONS OF RISAT TECHNIQUE

The technique of Restricted Immune System via Adoptive Transfer (RISAT) was utilized to drive an antibody response specific to a single T cell clone. A single T cell clone is adoptively transferred into an immunologically compatible immunodeficient mouse followed by an adoptive transfer of an open repertoire of immunologically compatible B cells. The RISAT animal is then immunized with antigenic protein preparations to elicit an immune response from both the T and B cells. B cells that receive T cell help will isotype switch to express IgG antibodies. These IgG antibodies recognize only antigen that both the B cell and the single T cell clone bind and can be used to identify the T cell's antigen.

There are still a number of diabetogenic T cell clones from the NOD mice with unknown antigens. The BDC-6.9 T cell clone was developed with the BDC-2.5 T cell. RISAT is being utilized to identify candidate autoantigens for the clone. Preliminary results show antibodies that detect protein bands at 55 and 33 kDa (unpublished data).

The simplicity of the RISAT system allows for it to be utilized in various disease models. RISAT would allow for the identification of a primary T cell clone's antigen without the expensive and time intensive techniques currently used, such as peptide or phage library screening (130, 131). Additionally, antibodies produced in RISAT animals can be utilized in biochemical assays such as immunoprecipitations and immunoblots. Skin lesions caused by infiltrating leukocytes characterize lupus. The MRL.lpr mice spontaneously develop skin lesions between 3 and 6 months of age (138). T cells isolated and expanded form these skin lesions can be used for RISAT in an immunologically compatible immunodeficient mouse. RISAT mice would be immunized using skin cells from the lesion site. The resulting antibodies would then be used to identify the isolated T cell antigen and expression patterns of the antigen through immunofluoresence, in addition to immunoblots to determine the antigen's expression in other tissues.

6.4 FUTURE DIRECTIONS FOR GRP78 AND TYPE 1 DIABETES

To further confirm that GRP78 is the antigen for the BDC-2.5 T cell clone, additional BDC-2.5 T cell assays with purified GRP78 from thapsigargin treated NIT-1 cells should be performed. Results should be correlated with silver stain and immunoblot to determine the purity of the purified GRP78. Furter, if *in vitro* serine phosphorylation of non-antigenic purified GRP78 causes these preparations to become antigenic that would provide strong evidence for the role of phosphorylation in the neo-antigenic of GRP78.

Identification of GRP78 antibody epitopes will provide insight on how GRP78 becomes an autoantigen. Immunoprecipitations with the BDC-2.5 TCR Tg serum demonstrate that sites

of phosphoserine are important to the recognition of GRP78 by the antibodies. Additionally, binding sites of commercially available GRP78 antibodies are blocked by thapsigargin treatment (**Figure 5.4**). Epitope mapping of the commercial antibody-binding sites will allow us to target a smaller region of the protein. The existing peptide library shown in **Table 5.I** and **5.II** can be used to epitope map the binding sites of the commercial antibodies as these antibodies are to unmodified peptide. Briefly, epitope mapping can be conducted by immobilizing peptide pools to wells of an ELISA plate. Commercial antibodies will be added to the well and allowed to bind to the immobilized peptides. A secondary antibody will be used to detect which peptide pools the commercial antibodies are able to bind.

As the T cell initiates the antigen-driven immune response, identification of the epitope responsible for the T cell response will allow for potential therapeutic targets. With the identification of the commercial antibodies' binding sites, there will be a limited number of peptides to further explore as candidate BDC-2.5 T cell epitopes. These candidate peptides should be chemically modified to contain phosphoserine as this PTM is specific to a subset of GRP78 and then tested against the BDC-2.5 T cell clone to determine which candidate peptides contain the T cell epitope.

The expression pattern of GRP78 should be determined, as this will provide information on the pathogenesis of how GRP78 is seen by the immune system. It has been reported that GRP78 acts as a surface receptor. If GRP78 is localized on the surface of the cells, this could be the mechanism that allows the immune system to recognize the resident ER protein. The expression patterns of GRP78 in different mouse strains, i.e. NOD, ALR, NOR, are of particular interest. Immunofluoresence analysis can be conducted with the BDC-2.5 TCR Tg serum. Additionally, it remains to be seen whether GRP78 is an autoantigen for human T1D. Screening

of T1D patients at various stages, i.e. recent-onset, 1, and 5 years post diagnosis, and at-risk first degree relatives for autoantibodies and autoreactive T cells to unmodified and modified GRP78 is critical to show the clinical relevance of my findings.

The diabetes incidence is striking different in the BDC-2.5 TCR-Tg depending on the background which it is bred onto. On the NOD background, only 10-15% of BDC-2.5/NOD animals spontaneously develop diabetes. By 3-5 weeks of age 100% of BDC-2.5 mice bred to the NOD.*scid* background develop diabetes (63). The BDC-2.5/B6^{g7} begins to develop diabetes at 3 weeks of age, with the average onset at 6 weeks. The diabetes incidence at 10 weeks is approximately 60% in the BDC-2.5/B6^{g7}. BDC-2.5/B6^{g7} animals that were free of diabetes at 10 weeks of age remain resistant (64). It would be interesting to determine if the B cell epitope is conserved in the different backgrounds.

The complexity of trying to decrease GRP78 levels will make therapies directed at GRP78 expression less than ideal. In attempts to deplete GRP78 with dsRNAi, Quian et al failed to completely deplete GRP78 protein levels (139). There is a feedback mechanism that regulates GRP78. I encountered this when I tried to block GRP78 expression with siRNA. Immunoblots showed that the siRNA treatments actually upregulated GRP78, which correlated with increase IFN-γ production by the BDC-2.5 T cell clone when incubated with siRNA treated NIT-1 cells (data not shown). Additionally, GRP78$^{-/-}$ (knockout) mice are embryonic lethal demonstrating how essential GRP78 is for cell survival (140). Therapies that quell ER stress may have success, since the activation of ER stress is what leads to increased and modified GRP78, thus promoting antigenic recognition by the immune system.

Although, the window of disease transfer with the BDC-2.5 T cell clone is early in postnatal development, the BDC-2.5 T cell clone has not been demonstrated to be the initiating T

cell for development of T1D. Strategies to remove the BDC-2.5 T cell clone from the NOD mouse and determine if diabetes onset is altered should be explored to identify the relevance of GRP78 as an initiating antigen in T1D. Use of the tetramer antibody that binds to the TCR of the BDC-2.5 T cell may be one way to selectively eliminate the BDC-2.5 T cell. Complexing of FasL to the tetramer antibody could result in the selectively killing of the BDC-2.5 T cell allowing for the assessment of how diabetes progressions in the absence of the BDC-2.5 T cell.

If GRP78 is shown to be an initiating antigen, vaccine development against this protein should yield protection against the development of T1D. As improvements are made in identifying individuals that will become diabetic, a vaccine to build tolerance against early autoantigens would be beneficial. A vaccine for GRP78 could potentially be used for other autoimmune diseases as RA, MS, and lupus have been shown to have GRP78 specific T cells (83, 125) (141).

6.5 FINDINGS OF THE THESIS

In conclusion, the work presented in this thesis has demonstrated the following:

(1) BDC-2.5 T cell drives antibody production to GRP78 both in the novel technique RISAT and in the BDC-2.5 TCR Tg mouse model.

(2) The BDC-2.5 TCR-Tg antibody recognizes a subset of GRP78, which likely contains the posttranslational modification, phosphorylation, specifically serine phosphorylation.

(3) Additionally, after induction of ER stress and upregulation of GRP78 by thapsigargin treatment, the non-antigenic NIT-1 cell line is able to initiate IFN-γ production from the BDC-2.5 T cell clone.

(4) The role of ER stress, GRP78, in the development of T1D is beginning to be elucidated and may be shown to be the common link between the various environmental triggers and the progression to T1D.

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