

**HIGHER LEVELS OF ER STRESS IN DIABETES-PRONE RATS
PLAYS A ROLE IN APOPTOTIC SIGNALING**

A Major Qualifying Project Report

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ABSTRACT

Biobreeding diabetes-prone (BBDP) rats exhibit a severe T cell lymphopenia, which may be linked to cellular stress. Expression of GRP78, an ER stress regulatory protein, was examined in various T cell populations of BBDP and BioBreeding diabetes-resistant rats (BBDR). Flow cytometry analysis revealed increased ER stress in all mature populations of T cells examined from the BBDP rat, but not in the immature thymocytes. These results suggest that increased ER stress in BBDP rats may play a role in apoptotic signaling that leads to T cell lymphopenia.

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BACKGROUND

Diabetes Mellitus

Diabetes, the disease the International Diabetes Federation calls the epidemic of the 21st century, affects close to 200 million people worldwide, and is the fourth leading cause of death in most developed countries (Diabetes Atlas 2006). In 2005, an estimated 1.1 million people worldwide died from diabetes, and it is predicted that diabetes deaths will increase by more than 50% in the next 10 years. In the United States diabetes affects over 20 million people, or about 7% of the population. Unfortunately, nearly one-third of those affected by diabetes are unaware they even have the disease.

General Description

Diabetes is a chronic disease in which the body does not produce or properly use insulin, a protein that is necessary to convert sugar, starches and other food into energy. Insulin, secreted by cells in the pancreas, is primarily involved in cellular glucose uptake. There are two main types of diabetes; Type I and Type II. Type I diabetes results when the cells that make insulin are destroyed, and are thus unable to produce insulin. Patients with type I diabetes also exhibit a severe T cell lymphopenia, a decreased number of T cells in the immune system. Type II diabetes results from tissue resistance to insulin, meaning their insulin receptors and subsequent signal transduction pathways are non-responsive. The focus of this project is T cell lymphopenia in type I diabetes mellitus (T1D).

Type I Diabetes Mellitus (T1D)

As stated above, type I diabetes is caused by the pancreas's inability to produce insulin, a polypeptide hormone responsible for regulating the amount of sugar in the bloodstream. It is produced naturally by β cells of the Islets of Langerhans in the pancreas. The pancreas secretes insulin in response to sugars being absorbed from the intestines into the bloodstream. Insulin acts to promote the cellular uptake of blood glucose, predominately by upregulating the expression of glucose transporters (GLUT) and therefore lowers the blood glucose concentration (Figure 1).

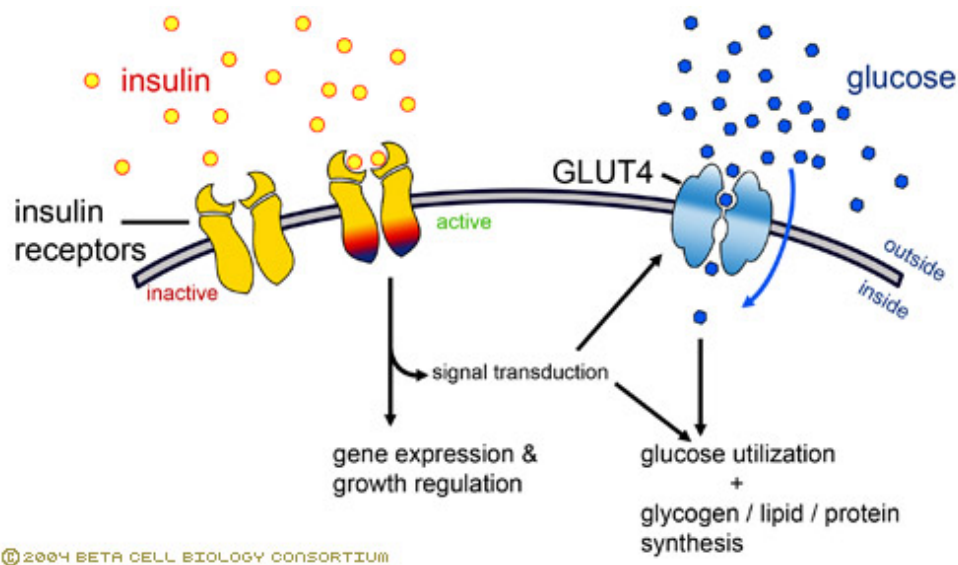


Figure 1. Insulin-Mediated Glucose Uptake. Insulin binds to the insulin receptor on a cell that induces a signal transduction cascade and allows the glucose transporter to transport glucose into the cell. (Beta Cell Biology 2007)

In T1D, the β cells of the islets are destroyed and unable to make insulin. The effects of this are associated with elevated blood glucose levels of affected individuals, and although blood glucose levels are elevated the cells are unable to uptake glucose due to the lack of insulin. The most common symptoms of diabetes include polyuria (frequent urination), polydipsia (excessive thirst) and polyphagia (increased appetite).

When the glucose concentration in the blood is high, incomplete re-absorption of glucose in the kidney occurs and some of the glucose remains in the urine (glycosuria). This inhibits the uptake of water by the kidney and results in increased urine production (polyuria). In turn this results in increased fluid loss causing dehydration and excessive thirst (polydipsia). (Lapen 2006)

Other symptoms of diabetes include significant weight loss, despite an increase in appetite and fatigue. Since the cells cannot utilize glucose the body uses fat and protein as alternative energy sources. This results in both a decrease in fat and muscle, and an increase in fatigue (Lidstone, 2005). Additionally, if left unchecked T1D can be fatal. This breakdown of fat and muscle results in byproducts called ketones that accumulate in the blood and cause high blood acid levels (acidosis). This results in a condition called diabetic ketoacidosis (DKA), which can lead to coma and even death. (Matthews 2003)

Since T1D is a chronic, lifelong, disease, a lot of time and money is dedicated to treatment of the disease. Currently, the most common and effective treatment of T1D is insulin replacement therapy. Although the ideal therapy would be a closed implant system capable of regulating blood glucose by producing insulin similar to the pancreas, this has yet to be perfected. Several published experiments using implants have successfully achieved normoglycemia in diabetic mouse models (see below), but similar implants have not yet been implemented in humans. Instead in patients subcutaneous injections of insulin or an insulin pump are used. Insulin isolated and purified from cow and pig pancreata was first used for therapy. However, with the development of recombinant DNA technology, insulin is now biomanufactured from *E. coli* bacteria. (Lidstone 2005)

In addition to the development of new types of insulin, a lot of research is currently being performed involving islet transplantations in both mice and humans. Islet transplantations involve implanting live pancreatic islets from either animals or from the pancreas of a deceased organ donor into a patient with T1D. These new islets immediately begin making insulin and mimic the effects of insulin in non-diabetic individuals. Nevertheless, as with any transplant, rejection has been a major problem. In most cases the autoimmune response that destroyed the patients own islets in the first place, also destroys the transplanted islets. However, trans-gene therapies, and a combination of immunosuppressive and anti-rejection drugs are currently being researched to halt the autoimmune response. (NDIC 2007)

BioHybrid Technologies, Inc. in Shrewsbury, MA have also created a biohybrid perfused artificial pancreas. It is designed to incorporate both islet tissue and a selective membrane that isolates this tissue from the immune system of the recipient. Biohybrid pancreas devices containing canine islet allografts were implanted in ten dogs requiring 18 to 32 units of injected insulin daily. These implants resulted in good control of blood glucose levels in six of these animals without further exogenous insulin for periods of up to 5 months (Sullivan *et al.* 2004). BioHybrid Technologies Inc. is hoping to eventually use this technology to replace current insulin therapy in humans, and provide a more precise regulation of blood glucose levels.

Type I Diabetes & Autoimmunity

Diabetes is among one of the most researched diseases in the world. Yet many questions relating to type I diabetes mellitus remain unanswered. Although at present much is known about the disease, the exact mechanism leading to T1D is not yet known.

The most commonly accepted hypothesis is that the immune system is triggered to destroy pancreatic β cells, causing a deficiency in insulin and resulting in hyperglycemia (high blood sugar).

The immune system is a complex collection of cells and molecules within an organism that serves many functions. It is composed of many interdependent cell types that collectively protect the body from infection. However, the heart of the immune system resides in the lymphocytes. Lymphocytes are a class of white blood cells that are made from hematopoietic stem cells in the bone marrow. Some lymphocytes, called B cells, remain in the bone marrow, are processed there, and then migrate to other areas of the body. Other lymphocytes, called T cells, leave the bone marrow and are processed in the thymus. Both types of lymphocytes play different roles in the immune system, although they often work together and influence one another's functions. This project involved various populations of T cells, including thymocytes, recent thymic emigrants and mature T cells. (Linnemeyer 1993)

As stated above, T cells are derived in bone marrow and then migrate to the thymus for differentiation. Immature T cells that travel from bone marrow to the thymus are called thymocytes, and those that are circulating in the bloodstream and lymph are called mature T cells. There are two major classes of T cells produced in the thymus: helper T cells, also known as $CD4^+$ T cells, and cytotoxic killer T cells, also known as $CD8^+$ T cells. The main functions of helper T cells are to stimulate B cells to make antibodies, proteins that recognize and neutralize foreign objects, and to activate cytotoxic T cells. Cytotoxic T cells recognize and destroy cells infected with viruses or

other pathogens. Both types of T cells can be found throughout the body, but they mostly reside in the lymphoid organs such as spleen and lymph nodes. (Linnemeyer 2003)

During an immune response, T cells recognize “non-self” antigens, macromolecules that elicit an immune response, on the surface of cells that have been processed and presented in combination with a “self” receptor called a major histocompatibility complex (MHC) molecule. MHC molecules are anchored in the cell membrane and display short polypeptides to T cells via the T cell receptors (TCRs). These polypeptides may be “self”, originating from a protein created by the organism itself, or they may be foreign, “non-self”, originating from viruses or other pathogens. (Abraham *et al.* 2001)

Recently it has been hypothesized that MHC’s may play a role in immune system attack against β cells in Type 1 Diabetes. It is known that $CD4^+$ T cells play a dominant role in the development of T1D and they recognize peptides presented on MHC class II molecules. Recent studies have shown that genes of MHC class II molecules are associated with susceptibility to T1D. It is believed that autoimmunity against β cells results, in a patient with T1D when MHC class II molecules have trouble defining “self” from “non-self” antigens. Therefore they bind to any antigen and stimulate an immune response on that antigen whether it is “self” or “non-self”. (Abraham *et al.* 2001)

There are many pathways in which autoimmunity can be triggered and the role of MHC’s is just one hypothesis. In addition to genetic variation of MHC’s, an over-sensitized T cell can mistake a “self” protein or polypeptide for a “non-self” one. This would also initiate an immune response against the “self” cell. (Lidstone 2005) The main course of events in diabetes pathogenesis is shown below in Figure 2.

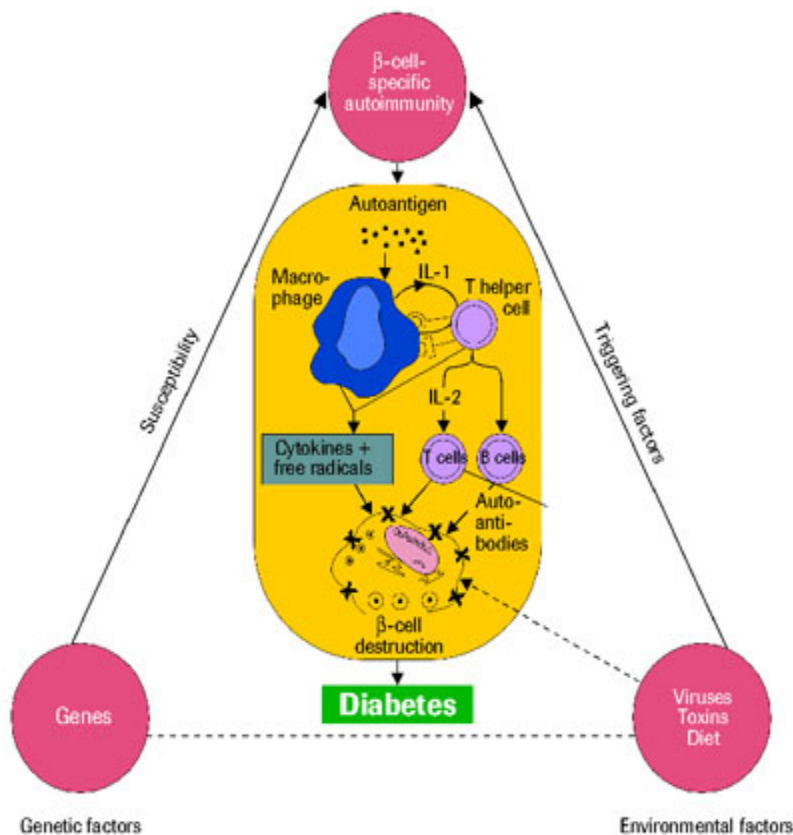


Figure 2. The Autoimmune Response in Diabetes. Due to either genetic (lower left) or environmental factors (lower right), auto antigens on β cells are recognized by cytotoxic T cells (upper center) which then lead to β cell destruction and T1D. (Roche Diagnostics 2007)

Apoptosis in Type I Diabetes

Apoptosis, or programmed cell death, has been shown to associate with many autoimmune diseases, including T1D. As stated above, it is hypothesized that pre-mature apoptosis of pancreatic β cells is the main cause of T1D. Although it is known that apoptosis is related to T1D the exact mechanism is still unknown.

Apoptosis often occurs when a cell is damaged beyond repair, infected with a virus, or when undergoing stress conditions. Cytotoxic T cells can also directly induce apoptosis in cells. The apoptotic mechanism is controlled by a diverse signaling pathway, involving a variety of caspase enzymes that eventually lead to DNA fragmentation,

nuclear blebbing, and cell death. Many different conditions or signals cause activation of the apoptotic pathway.

Determining the role of apoptosis in T1D has thus far been difficult. There are various theories regarding this issue in current research, and until recently it wasn't proven that β cell apoptosis was the primary cause of the disease. Now much research is dedicated to investigating apoptosis in β cells and to developing more effective treatments. The diagram in Figure 3 demonstrates apoptosis as the primary cause of type I diabetes. (Lidstone 2005)

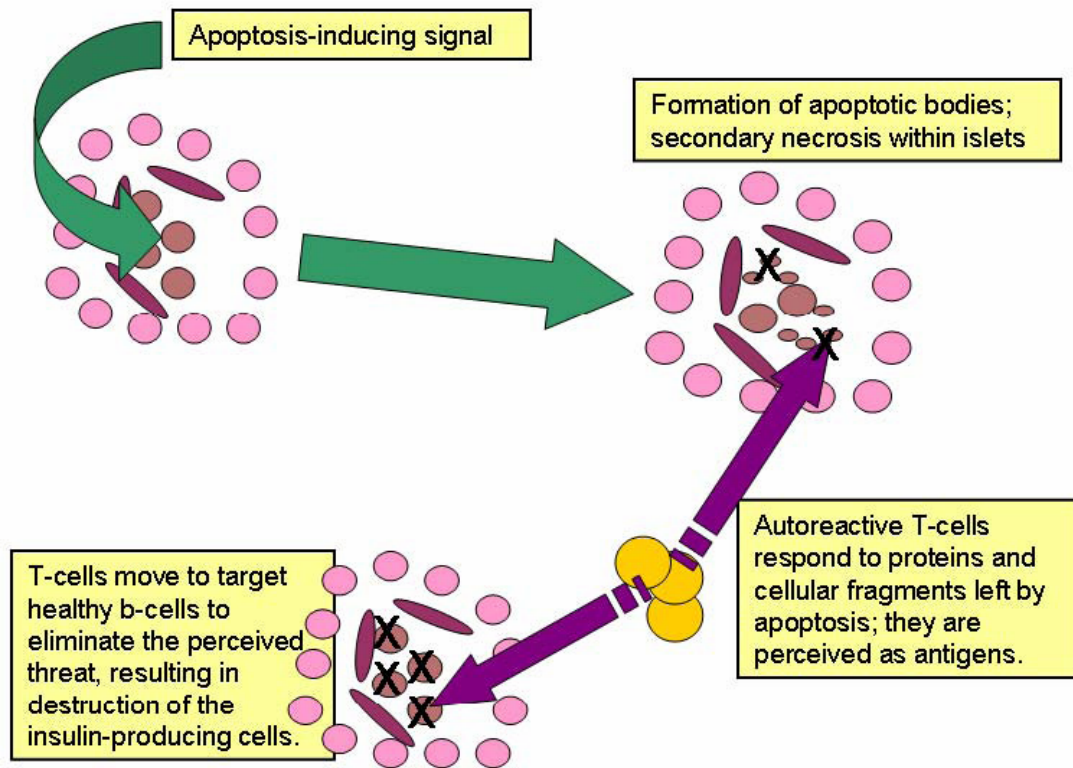


Figure 3. Apoptosis as a Primary Cause of Diabetes. It is hypothesized that pancreatic β -cell apoptosis may primarily be responsible for the autoimmune response characteristic of diabetes. Apoptotic bodies or proteins left by secondary necrosis initiate an immune response that results in islet cell destruction, effectively eliminating the production of insulin. (Lidstone 2005)

T Cell Lymphopenia

Lymphopenia is a condition marked by abnormally low levels of lymphocytes in the blood. Lymphocytes normally account for 15-40% of all white blood cells in the bloodstream. This condition is often detected when blood tests are performed to diagnose other diseases. Because of this, it is very hard to treat, and normally treatment is designed to identify and correct the underlying cause of the condition.

In diabetes, an abnormally low number of T lymphocytes are present. This T cell lymphopenia is characterized by a reduction of both CD4⁺ and CD8⁺ T cells in the periphery. In addition, studies have shown that in BBDP rats, a mutation in the *Gimap5* gene, also known as the *Ian4* gene, is responsible for the T cell lymphopenia that leads to diabetes development (MacMurray *et al.* 2002).

Gimap5 Mutation

The *Gimap* gene belongs to a family of integral membrane proteins localized to the mitochondria. They exhibit GTPase activity, are found in mice, rats, plants and humans and are a novel member of the Immune-Associated Nucleotide-related gene family. Many studies link members of this gene family to regulation of apoptosis. Apoptosis is regulated by both extrinsic and intrinsic signaling pathways. Recently, a frameshift mutation in *Gimap5* has been shown to contribute to T cell apoptosis through the intrinsic pathway by instigating permeabilization of mitochondria and releasing apoptogenic factors. (Pandarpurkar *et al.* 2003)

The absence of *Gimap5* in T cells has been shown to lead to mitochondrial dysfunction and has been associated with the expression of diabetes. It is believed that the loss of regulatory T cells is due to the *Gimap5* mutation, which may lead to an

imbalance between regulatory T cells and autoreactive T cells. The remaining autoreactive T cells will then mount an immune response against the insulin producing β cells.

The absence of *Gimap5* in T cells has also been shown to cause the increased expression of various heat shock proteins, and an increased level of mitochondrial stress. Heat shock proteins are highly conserved proteins that function as molecular chaperones in a cell. Expression of these proteins is increased during cellular stress including heat shock, infection, and oxygen deprivation. This increased expression is designed to prevent protein aggregation, enhance protein folding capabilities, and reestablish normal cellular homeostasis. (Pino 2005)

Endoplasmic Reticulum Stress

The endoplasmic reticulum (ER) is a multifunctional organelle involved in protein translation, folding, and transport to the secretory pathway or to the extra cellular environment. Because of its importance, the ER has an efficient system, consisting of protein chaperones and several other “quality control” mechanisms, that prevent the accumulation of unfolded or aggregated proteins, and corrects misfolded proteins. Collectively, this system is termed the unfolded protein response (UPR). (Kaufman 2002)

The accumulation of misfolded or aggregated proteins causes ER stress which then leads to the activation of the UPR (Figure 4). Therefore, the UPR is a stress-induced signaling cascade that when activated reduces the number of new proteins entering the ER lumen, increases degradation of ER-localized proteins and reinforces the protein folding capacity of the ER. Three proteins are known to act as key transducers of the UPR; activating transcription factor 6 (ATF6), inositol-requiring phenotype (IRE1) and

double-stranded RNA activated kinase-like ER kinase (PERK). Each of these proteins is expressed in all cells, localized to the ER, and is most importantly bound to ER chaperone Ig binding protein (BiP; also known as glucose-regulated protein 78). (Gass *et al.* 2004)

BiP/GRP78, glucose related protein, is the most abundant ER chaperone and a focus of this MQP. GRP78 promotes protein folding and prevents aggregation of proteins using the energy from ATP hydrolysis. When the ER is undergoing stress, GRP78 is released from ATF6, IRE1 and PERK. It is proposed that the unfolded proteins bind to GRP78 inhibiting it from interacting with the three transducers and in turn activating them. The three proteins each act separately to limit protein-folding, and by reducing the number of new proteins entering the ER. Therefore, GRP78 negatively regulates the UPR because it is bound to all three UPR transducers under non-stressed conditions, and it releases from the three transducers under stressed conditions. (Gass *et al.* 2004)

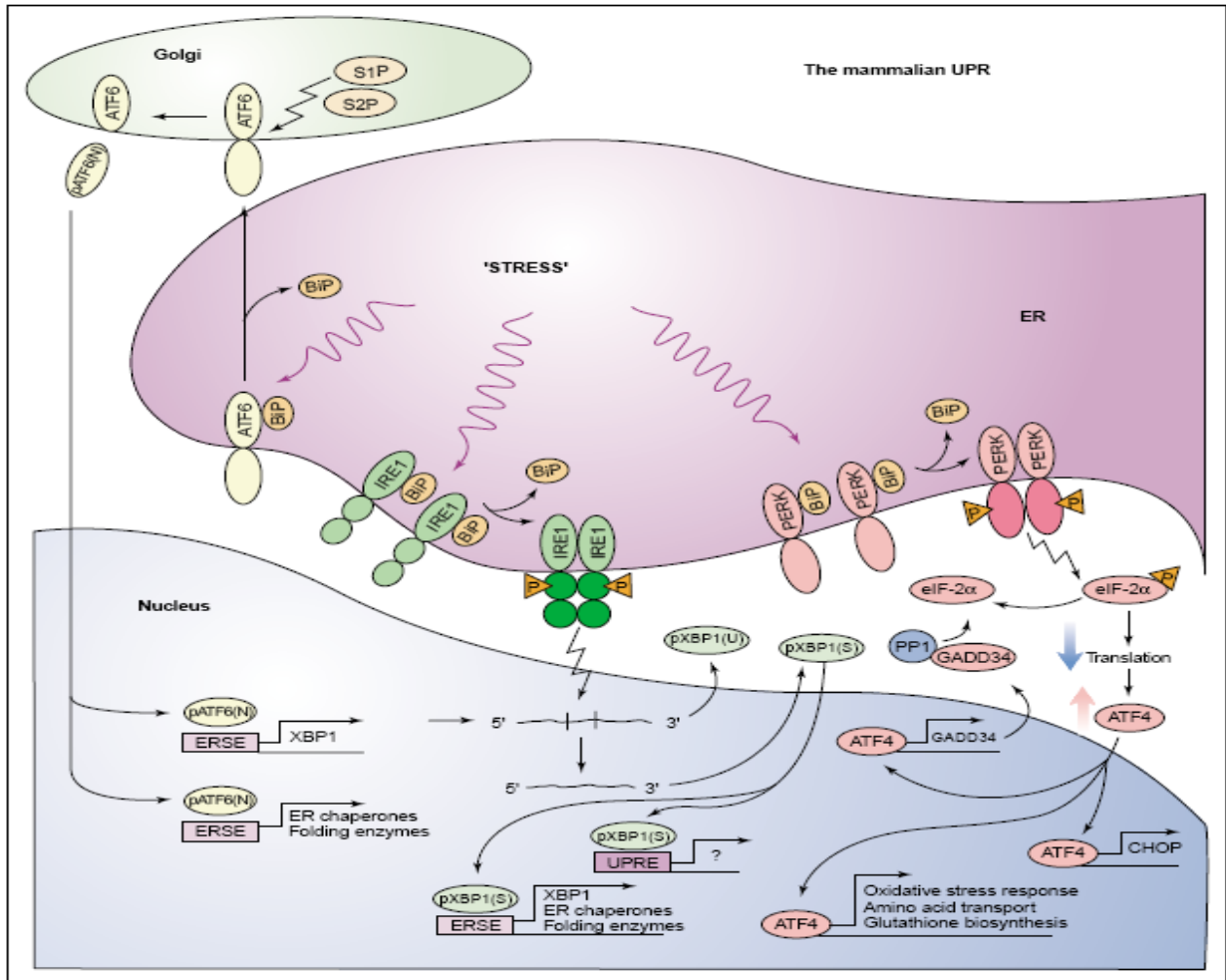


Figure 4. The Unfolded Protein Response. Upon stress BiP/GRP78 is released from the three transducers which work to alleviate unfolded proteins. (Gass *et al.* 2004)

Since cell stress and cell death are intimately linked, the upregulation of GRP78 in BBDP rats may be a key regulatory point in apoptosis (Garrido *et al.* 2001). In addition, an ER stress-mediated apoptotic pathway has emerged as a regulator of a variety of cell death stimuli by initiating signals from the ER (Pino 2005). This MQP will investigate the upregulation of GRP78 in various populations of T cells in BBDP and BBDR rats.

Animal Models for Diabetes

In medical research, the ideal model to study human disease would be humans, however, due to both ethical and complexity reasons, this is not always possible. Therefore, the second best model is used, animals. Animals are often convenient models for disease as they sometimes can be affected with human diseases. This provides scientists with a better understanding of the disease and disease mechanisms. In addition most animals, used in research, have a relatively short lifespan, and are reduced in size and living requirements (Lidstone 2005).

In diabetes research, mice and rats are the primary models used. Both models provide a large number of offspring, have life spans typically less than 5 years, and have demonstrated high levels of consistency when breeding for a certain trait. (Lidstone 2005) Two of the most common models used today are the non-obese diabetic (NOD) mouse and the BBDP rat.

The BioBreeding rat was discovered in 1974 at BioBreeding Laboratories in Canada and is the focus of this study. These rats spontaneously develop type I diabetes due to T cell-mediated autoimmune destruction of pancreatic islet β cells. These animals are described as “diabetes prone” BB rats as opposed to the “diabetes resistant” BB rats developed later. This model shares many areas of homology with humans and closely imitates human diabetes type I. (Mordes *et al.* 2001)

BBDP rats also exhibit a severe T cell lymphopenia and have a frameshift mutation in *Gimap5* (*Gimap5*^{-/-}). In contrast BBDR rats are *Gimap5*^{+/+}, non lymphopenic, and do not spontaneously develop autoimmune diabetes. These rats are used as a control

strain in demonstrating the difference between their diabetes prone counterparts. In this project the BioBreeding rats will be used to study levels of the stress protein, GRP78, in various populations of T cells.

PROJECT PURPOSE

The purpose of this MQP was to investigate the levels of cellular stress protein, GRP78, in various populations of T cells in BBDR and BBDP rats. Diabetes-prone rats exhibit a severe T cell lymphopenia, and because cell stress and cell death are intimately linked, cellular stress will be investigated in this project. Three populations of T cells will be investigated, thymocytes, recent thymic emigrants, and mature T cells. Flow cytometry will be used to look at how many cells are expressing GRP78 and at what level of intensity. This will provide valuable information regarding cellular stress of T cells. The hypothesis is that expression of GRP78 will be upregulated in T cells from BBDP rats and therefore cellular stress is occurring which may be a key regulator of apoptosis.

METHODS

Animals. BBDR and BBDP rats were obtained from Biomedical Research Models (Worcester, MA). Eight week-old male or female rats were used, and all of the rats were nondiabetic at the time of study. All animals were housed in a viral-antibody-free facility and maintained in accordance with the guidelines of the University of Massachusetts Medical School Institutional Animal Care and Use Committee and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

Thymus and Lymph Node T Cell Preparation. Cervical and mesenteric lymph nodes were removed from BBDR and BBDP rats and processed aseptically.

Antibodies. Anti-rat CD8a-PE (clone Ox-8), PerCp-conjugated anti-rat TCR $\alpha\beta$ (clone R73), anti-rat CD90-FITC (clone HIS51), Biotin-conjugated anti-rat CD25 (clone OX-39), and Streptavidin-conjugated APC-Cy7 mAbs were obtained from BD Pharmingen. Purified GRP78 mAbs (clone 40) and isotype control Abs were also obtained from BD Pharmingen. A Zenon Mouse IgG2a Labeling Kit, Alexa Fluor 647 (Invitrogen, Carlsbad, CA), was used to label purified GRP78 mAbs per the manufacturer's directions. Anti-rat CD4-Pacific Blue mAb and its corresponding isotype control Ab were obtained from Serotec.

Flow Cytometry. Single cell suspensions from cervical and mesenteric lymph node cells were washed and suspended in PBS containing 1% fetal clone serum (HyClone) and 0.1% sodium azide (Sigma-Aldrich). Samples were washed, and then incubated for 20 min with fluorescent mAbs to cell-surface markers as described in the text. To detect GRP78, cells were permeabilized using Cytofix/Cytoperm (BD Pharmingen) according to the manufacturer's directions. Cells were washed and incubated with Alexa Fluor 647-conjugated GRP78 mAb for 20 min. Labeled cells were washed, fixed with 1% paraformaldehyde (Polysciences, Warrington, PA) in PBS and analyzed with a LSR II (BD Biosciences, San Jose, CA) and FlowJo Software (PC version 5.5; Tree Star, Ashland, OR). Lymphoid cells were gated according to their light-scattering properties.

RESULTS

Diabetes-prone rats exhibit a severe T cell lymphopenia believed to be due to a frameshift mutation in the *Gimap5* gene. In addition, because cell stress and cell death are linked, levels of GRP78, an ER stress protein, were investigated in various populations of T cells in diabetes-prone and diabetes-resistant rats. T cell populations of thymocytes from the thymus, recent thymic emigrants, and mature T cells from the lymph nodes were investigated. Flow cytometry was used to compare GRP78 expression levels in various T cell populations in BBDR and BBDR rats.

Flow Cytometry

To be analyzed correctly, flow cytometry data requires a gate. Ungated samples contain a significant amount of cells other than lymphocytes (including macrophages, B cells, etc.). To correctly analyze the flow cytometry data, gates were set using forward and side scatter (X and Y axis, respectively) to limit the data analysis to lymphocytes in the sample. The gate for thymocytes in the thymus of BBDR and BBDR rats is shown in detail in Figure 5, with 92.46% and 93.75% of the cells being lymphocytes in the respective samples.

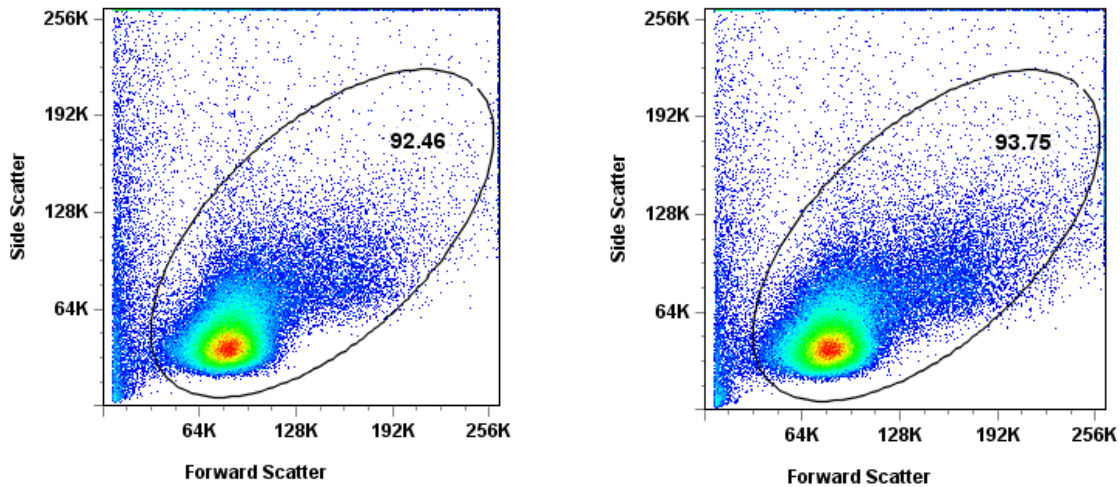


Figure 5. ThymocyteGating for Flow Cytometry Analysis of the Thymus. The figure on the left is the thymocyte gate for BBDR thymus, and the figure on the right is the thymocyte gate for BBDP thymus. Circled areas denote cells designated as lymphocytes via their forward scatter (X-axis) and side scatter (Y-axis).

Flow cytometry analysis of thymocytes (immature T-cells) in Figure 6 and Figure 7, reveals no difference between BBDR and BBDP rats, respectively. All populations of cells including $CD4^+$ cells (upper left quadrant), $CD8^+$ cells (bottom right quadrant), $CD4^+/CD8^+$ cells (upper right quadrant), and $CD4^-/CD8^-$ cells (bottom left quadrant) were similar for both the BBDR and BBDP rats. This data also shows that about 88% of the immature thymocyte cells have yet to be differentiated (presenting both CD4 and CD8) in the thymus in both BBDR and BBDP rats (upper right quadrant).

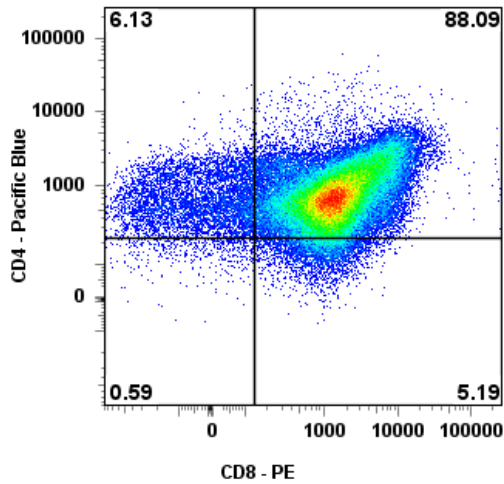


Figure 6. BBDR Thymus. Scatter plot of BBDR thymus illustrating normal amounts of double positive (CD4⁺/CD8⁺) thymocytes (upper right corner).

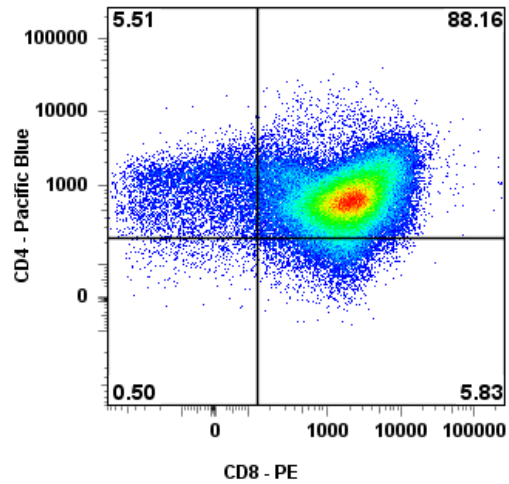


Figure 7. BBDP Thymus. Scatter plot of BBDP Thymus illustrating normal amounts of double positive (CD4⁺/CD8⁺) thymocytes (upper right corner).

Expression of GRP78 was analyzed in different thymocyte populations from the thymus: CD4⁺CD8⁺ thymocytes, CD4⁺CD8⁻ thymocytes and CD4⁻CD8⁺ thymocytes. In Figures 8-10 below, the y-axis is the number of cells expressing GRP78, and the x-axis is the level of GRP78 expression. Additionally, the dark blue line represents diabetes-prone rats, the solid tan represents the diabetes-resistant rats, and the violet dashed line represents negative control. As one can see in Figures 8-10 there is no difference in the expression of GRP78 between the diabetes-prone and diabetes-resistant rats in all population of thymocytes. The data for the thymus is shown above; the remaining data will be from the lymph nodes.

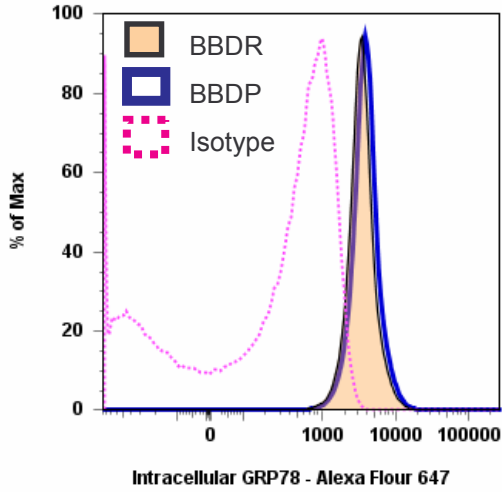


Figure 8. GRP78 Levels in CD4+CD8+ Thymocytes. Plot illustrating no difference in the levels of GRP78 between BBDR and BBDP rats.

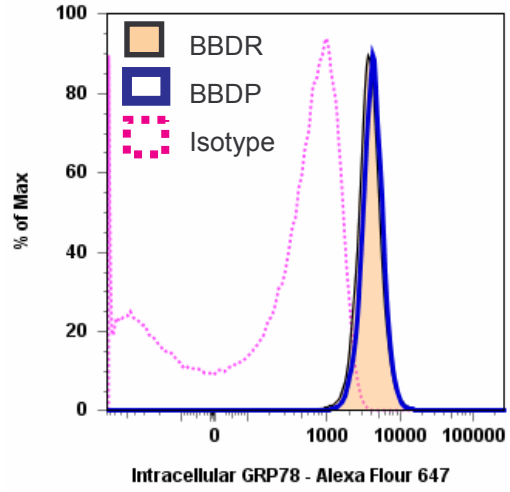


Figure 9. GRP78 Levels in CD+CD8- Thymocytes. Plot illustrating no difference in the levels of GRP78 between BBDR and BBDP rats.

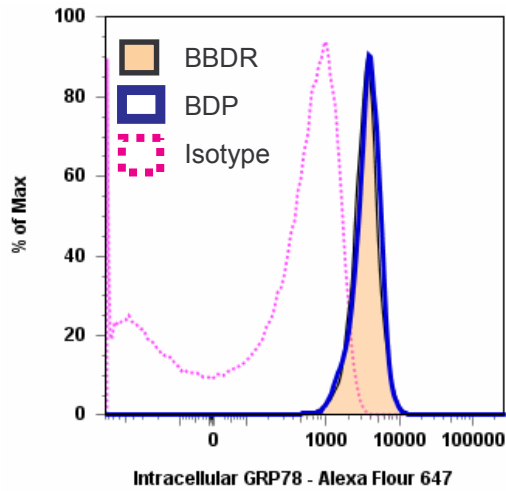


Figure 10. GRP78 Levels in CD4-CD8+ Thymocytes. Plot illustrating no difference in the levels of GRP78 between BBDR and BBDP rats.

The lymphocyte gate for the lymph nodes of BBDR and BBDP rats is shown in Figure 11. Setting gates allows for consistent data analysis.

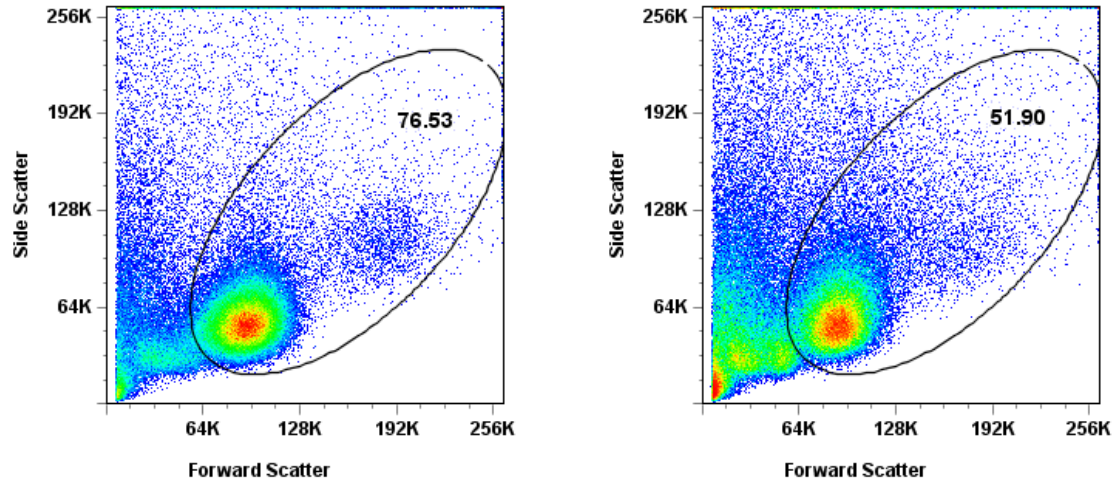


Figure 11. Lymphocyte Gating for Flow Cytometry Analysis of Lymph Nodes. The panel on the left is the lymphocyte gate for BBDR lymph nodes, and the panel on the right is the lymphocyte gate for BBDP lymph nodes.

It was evident above that T cell lymphopenia was not occurring in the thymus therefore, the lymph nodes, where T cells primarily reside, were investigated for lymphopenia. Figures 12-15 demonstrate that T cell lymphopenia occurs in diabetes-prone rats as compared to diabetes-resistant rats. In Figure 13, T cell lymphopenia is evident in the BBDP rat as almost 67% of cells are double negative ($CD4^-CD8^-$), bottom left corner. In addition Figure 15 demonstrates that only 21% of cells in the diabetes-prone rat are expressing T-cell Receptors (TCRs) as compared with 73% of cells expressing TCRs in the diabetes-resistant rat (Figure 14).

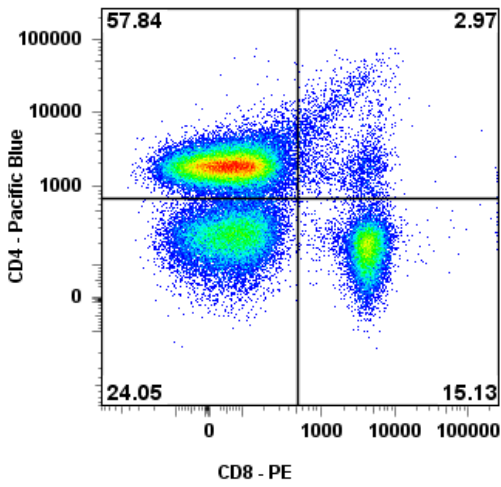


Figure 12. CD4 and CD8 Lymphocyte Numbers in BBDR Rat Lymph Nodes. Plot illustrating normal levels of CD4+ (upper left quadrant) and CD8+ cells (lower right quadrant) in BBDR rat lymph nodes.

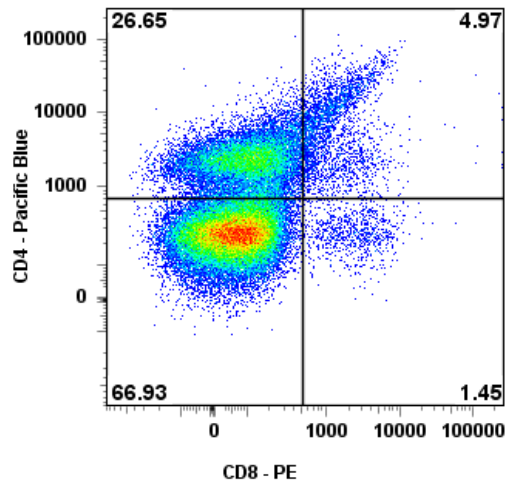


Figure 13. CD4 and CD8 Lymphocyte Numbers in BBDR Rat Lymph Nodes. Plot illustrating mature T cell lymphopenia (upper left and lower right quadrants) in the BBDR rat lymph nodes.

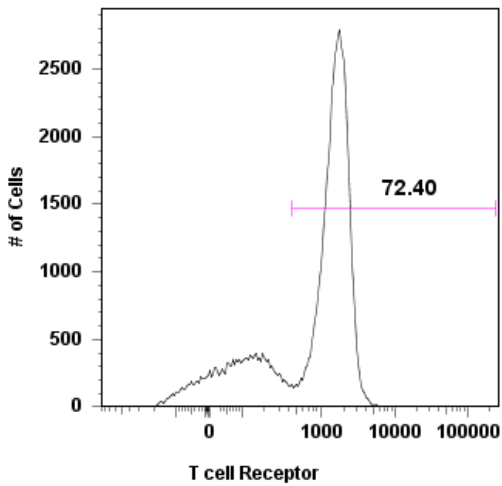


Figure 14. BBDR TCR. Plot illustrating normal numbers of cells expressing T cell receptors (72%) in the BBDR rat.

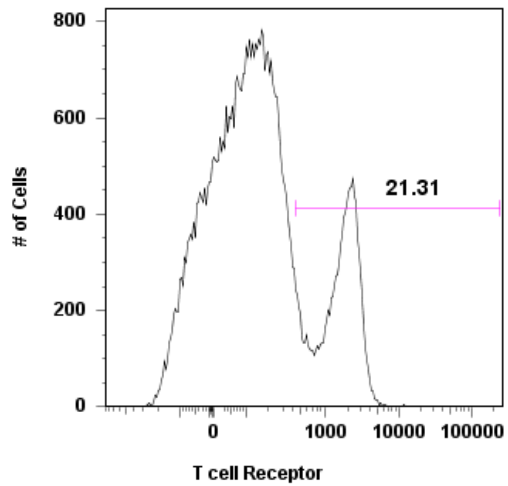


Figure 15. BBDR TCR. Plot illustrating T cell lymphopenia in BBDR rats, only 21% of cells expressing T cell receptors.

The second population of T cells that was analyzed is the recent thymic emigrants (RTEs). Recent thymic emigrants express CD90; therefore CD90⁺ was used as a marker. Figures 16-17 demonstrate a slightly increased level of GRP78 in CD4⁺ (left figure) and CD8⁺ (right figure) recent thymic emigrants of diabetes-prone rats as compared to diabetes-resistant rats. Because GRP78 is a stress protein, these results indicate that the recent thymic emigrants are beginning to undergo cellular stress.

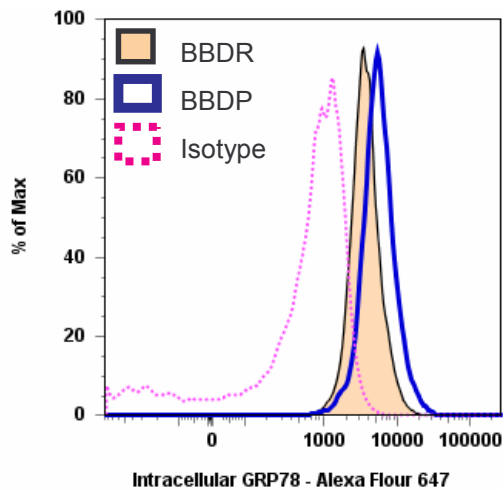


Figure 16. TCR⁺CD4⁺CD90⁺ RTEs.
Plot illustrating increased levels of GRP78 (shift to the right) in BBDP rats (blue line) opposed to BBDR rats (solid tan).

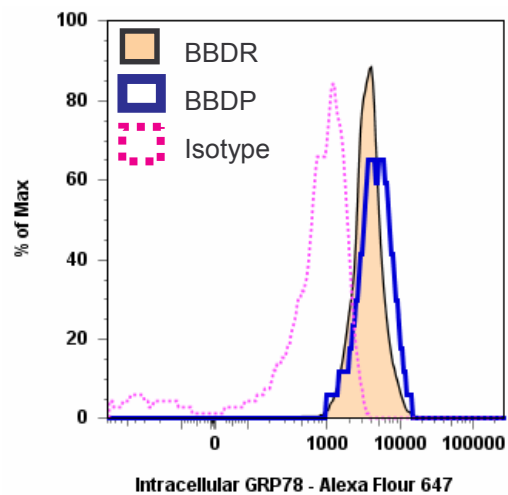


Figure 17. TCR⁺CD8⁺CD90⁺ RTEs.
Plot illustrating increased levels of GRP78 (shift to the right) in BBDP rats (blue line) opposed to BBDR rats (solid tan).

Finally, mature T cells were analyzed for increased levels of GRP78 in BBDP rats as opposed to BBDR rats. Mature T cells do not express CD90. Figures 18-19 demonstrate a slightly increased level of GRP78 in CD4⁺ (left figure) and CD8⁺ (right figure) mature T cells of diabetes-prone rats (dark blue line) as compared to diabetes-resistant rats (tan curve). Because GRP78 is a stress protein, these results indicate that

mature T cells are also beginning to undergo some stress. In addition mature T cells had an increased expression of GRP78 as compared with recent thymic emigrants (Figures 16-17).

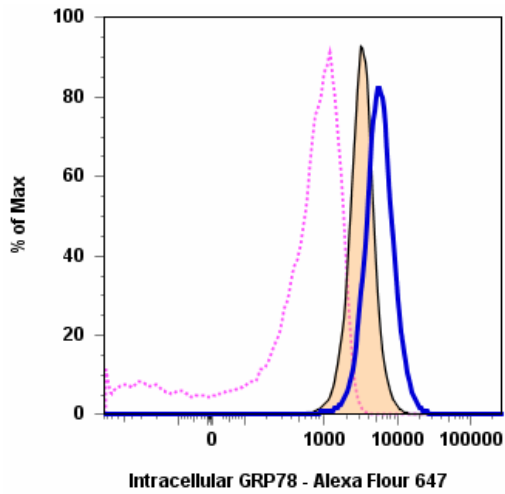


Figure 18. TCR⁺CD4⁺CD90⁻ Mature T cells. Plot illustrating increased expression of GRP78 in BBDP (blue line) versus BBDR (solid tan) rats.

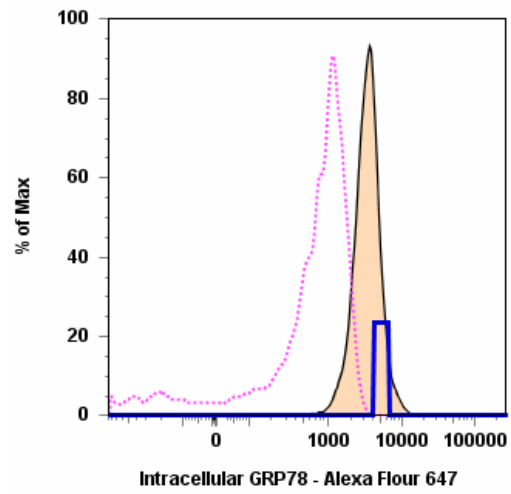


Figure 19. TCR⁺CD8⁺CD90⁻ Mature T cells. Plot illustrating increased expression of GRP78 in BBDP (blue line versus) BBDR (solid tan) rats.

DISCUSSION

From the flow cytometry analysis of diabetes-resistant and diabetes-prone rats, a few conclusions can be drawn. First, analysis of the thymus containing immature T-cells reveals no significant difference between diabetes-prone and diabetes-resistant rats, and no T cell lymphopenia is evident. In both cases about 88% of thymocytes were double positives ($CD4^+CD8^+$) as can be seen in Figures 6-7. Additionally, both BBDR and BBDP rat thymocytes revealed similar expression of stress protein GRP78, Figures 8-9. This indicates that cellular stress is not occurring yet in developing T cells in the thymus. Furthermore, because the *Gimap5* mutation (present in the BBDP rats) is linked to T cell lymphopenia, the mutation has yet to manifest itself in the thymus.

However, as expected, T cell lymphopenia is evident in the lymph nodes (containing mature T-cells) of diabetes-prone rats. This was evident in Figures 12-15 where diabetes-prone rats had few $CD4^+$ and $CD8^+$ T cells, and where very few cells expressed T cell receptors. Diabetes-resistant rats expressed three times as many T cell receptors as diabetes-prone rats. In addition, in the diabetes-prone rat, more than 65% of total cells detected by flow cytometry were other types of cells such as macrophages, B cells, etc. This indicates that the *Gimap5* mutation present in these BBDP rats most likely arises in the recent thymic emigrants, which causes cellular stress and ultimately cell death.

The main purpose of this project was to detect expression of GRP78, a stress indicating protein, in diabetes-resistant and diabetes-prone rats. It was hypothesized that the diabetes-prone rats would have increased levels of expression of GRP78. This was indeed found to be the case in recent thymic emigrants and in mature T cells. Figures 16-

19 demonstrate the increased expression of GRP78 in diabetes-prone rats. This data reveals that cellular stress is indeed occurring (or beginning to occur) in recent thymic emigrants and in mature T cells in BBDP rats. Because cell stress and cell death are intimately linked, cellular stress in BBDP rats may be contributing to the apoptosis of T cells, causing T cell lymphopenia.

Future research should be performed to further characterize the *Gimap5* mutation to determine if, indeed, the mutation leads to cellular stress which ultimately leads to cell death. For example, additional future experiments should include using siRNA technology to knockdown *Gimap5* in order to investigate the unfolded protein response and alternative cellular stress mediated apoptotic factors/pathways in BBDP T cells.

Although the results of this MQP can neither confirm nor deny that cellular stress and the *Gimap5* mutation are indeed the cause of T cell lymphopenia, a lot of valuable data was collected. Researchers now know that there is an increase in expression of GRP78, a valuable stress indicating protein, in diabetes-prone rats when compared to diabetes-resistant rats. Overall, this MQP has contributed to further characterizing both the *Gimap5* mutation found in BBDP rats and to researching cellular stress and its effects on cells.

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