10-2016

Bisphenol A Potentiates Development of Streptozotocin-Induced Type 1 Diabetes in an Experimental Mouse Model

Sinduja Thinamany
thsi0902@stcloudstate.edu

Bisphenol A Potentiates Development of Streptozotocin-Induced Type 1 Diabetes in an Experimental Mouse Model.

by

Sinduja Thinamany

A Thesis

Submitted to the Graduate Faculty of

St. Cloud State University

In Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in Cellular and Molecular Biology

October, 2016

Thesis Committee:
Marina Cetkovic-Cvrlje, Chairperson
Maureen Tubbiola
Nathan Winter

Abstract

Type 1 diabetes (T1D) has been historically, and continues to be the most common type of diabetes in children and adolescents, affecting nearly three million people in the Unites States. It is an autoimmune disorder characterized by the deficiency of insulin due to the T-cell-mediated autoimmune destruction of insulin-producing pancreatic beta cells. Strong advances have been made associating genetics to the development of T1D, however, an increase of disease incidence cannot be explained by genetic factors only, focusing attention to environmental triggers. Bisphenol A (BPA) is an endocrine disruptive environmental pollutant, found in plastic products, toys, and inner lining of can products. It is believed that BPA exposure is associated with adverse health effects in humans, such as obesity, type 2 diabetes, reproductive disturbances, and autoimmune diseases. However, the association of BPA and T1D has not been extensively scrutinized. The goal of this study was to investigate the effects of BPA on T1D development and its mechanism of action in the context of T-cells in a multiple low-dose streptozotocin (MLDSTZ)-induced T1D mouse model. It was hypothesized that BPA treatment of mice would increase diabetes incidence and potentiate the severity of disease through its alteration of T-cell populations and their function. To test this, four-weeks-old male C57BL/6 mice were exposed to 1 and 10 mg/L BPA dissolved in drinking water up to 16 weeks of age. To initiate the development of T1D, mice received five intraperitoneal injections of 40 mg/kg STZ at nine weeks of age. Glucose measurements were taken twice a week throughout the experiment. At the end of experimental period, on day 50 post first STZ injection, pancreata were analyzed for severity of mononuclear cell infiltration (insulitis). In addition, on day 11 and 50 post the first STZ injection, spleens were harvested and analyzed for composition, proliferation, and cytokine profiles of T-cells. In summary, the study showed that long-term exposure of MLDSTZ-treated C57BL/6J mice to low, as well as high BPA dose, potentiated diabetes development and insulitis severity. Whereas both BPA doses exhibited immunomodulatory activities, their diabetogenic mechanism of action appeared to be divergent. Thus, low-dose BPA exposure initiated diabetogenesis through the alteration of T-cell populations and skewing their function towards a proinflammatory Th1-like immune response early on during disease development, whereas high-dose BPA treatment did not show these effects. Overall, these results confirmed diabetogenic potential of BPA and its immunomodulatory effects on the T-cells, bringing awareness of BPA as a potential environmental trigger for the development of human T1D.

Acknowledgment

First and foremost, I would like to express my deepest gratitude to my mentor and research advisor, Dr. Marina Cetkovic-Cvrlje from Biology department, for being a constant source of knowledge, inspiration, and encouragement throughout this research. Not only did she give me an invaluable opportunity to join her research team as an undergraduate student and work my way up to being her graduate thesis student, she also provided academic advice and moral support throughout my time at St. Cloud State University. I am tremendously thankful for her faith in me, giving me a chance to work with her as the immunology research team lead, and countless conversations from which I learned more than I can put in words. Additionally, I am indebted to the remainder of my committee: Dr. Maureen Tubbiola and Dr. Nathan Winter, whose insightful comments helped me improve my research, and to Brian Lorenz, the vivarium manager for his assistance throughout my Master's program. I would like to convey my sincere gratitude to the Office of Sponsors Program for funding my project and giving me the wonderful opportunity to present at the annual research colloquium. I am also endlessly appreciative of alumni graduate assistants, Jordan Kuiper, Marin Olson, Cordelia Dunai, and talented undergraduate research students, not only for teaching me and constantly giving me suggestions, but also for their never-ending support and guidance over the past few years. Finally, I would like to express my deepest appreciation for my parents, siblings, and friends for being a continual source of moral support. Their faith and love, brought me success throughout my college career.

Table of Contents

Chapters **Page**

List of Tables

List of Tables

List of Figures

Chapter I- INTRODUCTION

The Immune System:

The roots of immunology and the study of immune system date back to 1796 when an English physician, Edward Jenner discovered a method of smallpox vaccination. Jenner noticed that people who were infected with cowpox were resistant to smallpox, where then he injected a young boy with substance from another person who had an active case of cowpox. After the boy recovered, Jenner then inoculated the boy with smallpox virus and found the boy was immune. Since then, many scientists have studied the amazing function of human body and its immune system and explained it as a network of cells, tissues, and organs that work together to protect the body from foreign particles that invades the body, whether they be pathogenic or non-pathogenic (National Institute of Allergies and Infectious Disease, 2016). Thus the immune system is known to be the defense mechanism of body against infection by pathogens, chemicals, and allergens and a key component in regulation of normal organismal homeostasis.

Only in the last century have the components and the cells of the immune system been discovered, and more remains to be clarified. It has been scrutinized that the achievement of the homeostasis is orchestrated by two main components of the immune system; innate and adaptive immunity. The innate immune system is the first to get invoked when the body encounters a foreign particle. It is not antigen-specific, and depends on certain proteins, natural killers, and phagocytic cells (macrophages, neutrophils, and dendritic cells). These cells express genetically encoded receptors called Toll-like receptors that recognize conserved features of a pathogen and gets quickly activated to fight against invaders.

The adaptive immune system, on the other hand, is an acquired immune system, and is

antigen-specific. It is, however, slow to develop on first exposure to a new pathogen due to the need for specific clones of B- and T-cells to be activated and expanded. Thus, it could take 5-7 days for the immune response to get effective. The adaptive immunity can be further divided into humoral and cell-mediated immunity that involves aforementioned B- and T-cells. B-cells are descendants of the hematopoietic stem cells that express specific antigen receptors, B-cell receptors, that are basically immunoglobulins or antibodies. Memory B-cells and plasma cells are two types of B-cells, which play a key role in initiating a secondary immune response when an organism is encountered by the same pathogen, and they are responsible for antibody production. The immune system branch that involves B-cells is also known as humoral immunity. In contrast, cell-mediated immunity involves the activation of T-cells and production of cytokines that help further amplification of the immune response. Via studying individual surface markers of T-cells, called clusters of differentiation (CD), different T-cell types can be identified as T helpers (Th) with expression of CD4 surface marker, T cytotoxic cells (Tc) with CD8 marker, and T regulatory cells (Treg) with both CD4 and CD25 surface markers, as well as intracellular identifier FoxP3. While the surface markers of each of the above mentioned T-cell type is different, the subtypes of Th cells, such as Th1, Th2, Th17 subsets, express the same surface marker CD4. These subsets are thought to arrive from a non-polarized naïve T0 precurser which could be differentiated into different T helper cells. These Th subtypes can be differentiated only by their cytokine secretion (University of Oxford, 2000).

Cytokines are proteins that direct the behavior of immune cells, which also help those immune cells to build an immune response when encountered with a pathogen or foreign particles in the body. Out of the aforementioned Th subsets, Th1 secretes IL-2 and IFN-γ, Th2

secretes IL-4 and IL-10, and Th17 secretes IL-17. IFN-γ plays a pivotal role in immune cell trafficking while, IL-2 activates the production of IFN-γ and aids in T-cell proliferation, differentitation, and growth. Th-17 exhibits anti-microbial properties and acts to potentiate autoimmunity (Wagner, 2011). TNF-α is another cytokine that is produced by macrophages, which aids in systemic inflammation, and is responsible for acute inflammation (Idriss & Naismith, 2000). In contrast, IL-4, a cytokine produced by Th2 subset, inhibits the differentiation of Th1 subset, and subsequently induces anti-inflammatory immune responses along with IL-10, which has similar functions as IL-4.

Complications arise when the immune system does not function properly. When the immune system is suppressed, immune deficiencies could occur. On the other hand, immune system can damage its own tissues, leading to either hypersensitivity or autoimmune disorders, such as multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, and type 1 diabetes mellitus (National Institute of Allergy & Infectious Disease, 2014).

Diabetes:

Diabetes is a collective term for several abnormal health conditions with variety of causes. The conditions of diabetes is reflected through high blood glucose level (hyperglycemia), and clinical conditions, including excessive urination, thirst, and unusual weight loss. The digestive tract breaks down the carbohydrate to glucose, a form of sugar that enters the bloodstream. Cells throughout the body absorb glucose with the aid of insulin, a metabolic hormone, and converts it to energy. Diabetes develops as a result of lack of insulin production or insulin resistance, a condition when the body cannot use insulin efficiently, leading to failure in controlling blood glucose level. The three common types of diabetes are: type 1 diabetes (T1D),

type 2 diabetes (T2D), and gestational diabetes. T2D accounts for 90-95% of all diagnosed diabetes cases (National Diabetes Report, 2014). It is a metabolic disorder in which, despite of the proper production of insulin, at least in the beginning of disease, the cells of the body, primarily muscle, liver, and fat tissues cells, cannot properly use the insulin (Silver, O'Neil, Sowers, and Park, 2011). The reason is insulin resistance, which develops as a consequence of the insulin receptor signal transduction dysfunction, and leads to glucose accumulation in the bloodstream. T2D has a strong association with obesity, and sedimentary lifestyle (United States National Library of Medicine, 2014). People with T2D can control their blood glucose level through proper diet, regular exercise, losing excess weight, and taking medications accordingly.

Gestational diabetes, as per definition, is a type of glucose intolerance diagnosed in pregnant women who are in their second or third trimester. It is a form of diabetes that could potentially harm both the mother and growing infant. Usually, once the infant is born, the mother recovers; in rare cases (5-10%), however, women with gestational diabetes continue to have high blood glucose levels, which lead to type 2 diabetes. It is also known that infants born to mothers with gestational diabetes have high risk of developing obesity and diabetes (Carwile and Michels, 2011).

Type 1 Diabetes:

The National Diabetes Statistics Report estimates that nearly 29.1 million people have diabetes in the U.S alone, of which approximately 3 million people have been diagnosed with T1D (American Diabetes Association, 2014). Although type 2 diabetes is increasingly diagnosed in youth, T1D has been historically, and continues to be the most common type of diabetes in children and adolescents (Maahs, West, Lawrence, & Davis, 2010). Data from large

epidemiologic studies worldwide indicate that the incidence of T1D has been increasing by 2-5% worldwide. Considering U.S, the prevalence of T1D is approximately 1 in 300 by 18 years of age (Maahs et al., 2010), and it has been shown that clinical trials and treatments to treat T1D cost US nearly 14.4 billion each year (Tao, Pietropaolo, Atkinso, Schatz, & Taylor, 2010).

Type 1 diabetes (T1D) is a T-cell dependent and T-cell mediated autoimmune disorder that is characterized by the absolute deficiency of insulin due to the autoimmune destruction of insulin-producing pancreatic beta cells. The onset of T1D is depicted by the increased blood glucose level, which is typically reflected through clinical conditions such as polyuria, polydipsia, polyphagia, and unusual weight loss (Maahs et al., 2010). Furthermore, the onset of T1D is proceeded by the inflammation of islets of Langerhans in the pancreas. The main lesion observed in the pancreas is a specific histologic lesion called insulitis, which is characterized by the accumulation of mononuclear cells,- including macrophages, dendritic cells, and T-cells in the pancreatic islets during the active killing of beta cells (Bodin, Bølling, Samuelsen, et al., 2013). The inflammation is initiated by autoantigens that mount an immune response against pancreatic beta cells (M. Lee, W. Lee, Todorov, & Liu, 2010). Some of the autoantigens that are responsible for initiating insulitis are glutamate decarboxylase 65 and 67, carboxypeptidase H, tyrosine phosphate-like proteins, and insulin itself. In a healthy individual, these autoantigens are recognized as “self” antigens by immune cells, which leads to no reaction of the immune system. In an individual with T1D, however, the immune cells recognize the aforementioned autoantigens as “foreign”, thus building an immune response against its own pancreatic beta cells that leads to their destruction (Roep and Peakman, 2012). Since T1D is a T-cell-dependant disease, one should study the T-cells, the immune cells of interest in this disease. There are

several different T-cell types which are involved in the etiopathogenesis of T1D.

It is essential to study the role these cells play in the progression of disease in both animals and humans to understand the mechanism of beta cell death in the pancreas. Out of the aforementioned Th subsets, Th1 and Th17 are considered to be pathogenic, while Th2 is considered to be protective towards T1D. Similarly, other T-cell types such as Tc and Treg can be classified as pathogenic and protective towards T1D, respectively (Sakaguchi, 2000). Tc cells are known to be involved in the direct killing of pancreatic beta cells, while Tregs are thought to be protective towards T1D by maintaining the homeostasis between pathogenic and protective immune responses. Cytokine production of these cells could be protective or pathogeneic against the autoimmune disorder, depending on the immune cells which secrete them.

While pathogeneic cytokines are known to promote activation of macrophages, the protective cytokines are known to inhibit the macrophage activation and promote Th2 and Treg protective immune responses, thus reducing beta-cell loss. Out of the aforementioned Th subsets, Th1 and Th17 secrete IL-2, IFN- γ and IL-17, respectively, which are considered pathogenic. In contrast, IL-4 and IL-10, secreted by Th2, are considered protective. These cytokines together counteract the pathogenic effects of Th1 and Th17 subsets, thus producing protective responses towardsT1D (Wagner, 2011). TNF-α is another pathogenic cytokine that is produced by macrophages and Th1-type cells (Idriss & Naismith, 2000).

Experimental Models of Type 1 Diabetes:

Considering the immune cells that play a vital role in disease development and genetic component of the disease, researchers have developed particular strains of inbred mice, which are the best animal models for studying T-cell-dependent mechanism(s) of T1D onset (Shafrir,

2007). Non Obese Diabetic (NOD) mice (Kikutani and Makino, 1980; Makino et al., 1980 & Delovitch and Singh, 1997), especially NOD females, spontaneously develop disease like humans. On the other hand, experimental T1D can be chemically-induced. For induction of T1D in animal models, two types of chemicals are commonly used - streptozotocin (STZ) and alloxan (a urea-derivative that induces selective necrosis in pancreatic beta cells) (Kolb-Bachofen, Epstein, Kiesel, & Kolb, 1988). BALB/cByJ and C57BL/6J mice represent common strains used for STZ-induced type of experimental T1D. These two mouse strains however, have differences in both innate (Watanabe, Numata, Ito, Matsukawa, & Takagi, 2004) and acquired immunity (Guinazu et al., 2004). Considering T1D, the two strains have inherited differences in the susceptibility to the effects of STZ in the development of hyperglycemia. It is known that C57BL/6J mice are more susceptible to the effects of STZ, having prominent Th1 immune response, which is pathogenic for T1D, than BALB/cByJ, who exhibit strong Th2 response, known as a protective one in pathogenesis of this disease (Herold, Vezys, Buckingham, & Baumann, 1997).

In order to create a T-cell-dependent autoimmune model of T1D, male C57BL/6 mice are treated with multiple low-doses of streptozotocin (MLDS). STZ is an antibiotic and anti-cancer drug. It is a synthetic nitrosoureido glucopyranose derivative isolated from fermentations of *streptomyces acromogenes*. STZ's structure is very similar to that of glucose, thus its high affinity towards pancreatic beta cells damages them, through which it aids in the potentiation of T1D (Utuk, 2010).

MLDSTZ administration has an ability to induce T1D in a particular mouse strain of a particular sex, such as C57BL/6J males. Females, however, have shown less susceptibility to

MLDSTZ-induced T1D (Utuk, 2010). The critical factor that determines the susceptibility of mice to the hyperglycemic effects of STZ is the relative overall level of androgens over estrogens that the male C57BL/6J mice display (Paik, Michelis, Kim, & Shin, 1982). Unlike higher doses of STZ, the multiple small doses of STZ, used for the induction of MLDSTZ model of T1D, are not believed to be directly toxic to beta cells. MLDSTZ induces an immune and inflammatory reaction related to the release of one of the autoantigens, glutamic acid decarboxylase. Under this condition, infiltration of ononuclear cells occurs in the islets of Langerhans, leading to the destruction of pancreatic beta cells (Graham et al., 2012).

Environment and Type 1 Diabetes:

Research on factors that influence the development of T1D has been an active area in the recent years with the hope to identify the causes that could potentially be targeted for intervention. Age, gender, race, genotype, geographic location, and seasonality are some of the factors that influence the development of TID (Maahs et al., 2010). Despite the findings of global variations in the incidence, prevalence, temporal trends, and risk factors of T1D by large registry studies, such as World Health Organization Multinational Project for Childhood Diabetes, known as the DIAMOND project, the complexity of this disease development has always been a roadblock to the attempt of finding the causes (Karvonen et al., 2000)

Though aforementioned T-cell types, subsets, and cytokines are considered to be responsible for the disease development, it is believed that T1D is instigated by the combination of both genetic and environmental factors. Researchers have studied the role of genetics in T1D, where multiple genes within the major histocompatibility (MHC) complex, among many other non-MHC-linked genes, have been discovered to be susceptibility loci linked with this disease

development (Rich et al., 2009). The role of non-genetic factors is evidenced by migration studies, rising incidence within genetically stable populations, and twin studies (Maahs et al., 2010); however, the role of environmental factors that trigger T1D remains largely unknown (Rich et al., 2009). Persistent organic pollutants (POPs) are considered to be a global issue, because of their high toxicity and inability to readily biodegrade. Numerous studies have been done in this arena that show associations of POPs to adverse health effects in humans, including autoimmune diseases (Richter et al., 2007).

POPs are organic compounds which were widely used during the boom in industrial production after World War II. Since then, thousands of synthetic chemicals were introduced into commercial use which have POPs in their backbone. Many of these chemicals proved beneficial in pest and disease control, crop production, and industry. These same chemicals, however, have had unforeseen effects on human health and the environment (United states Environmental Protection Agency [EPA], 2010). Due to this very fact, congress of the USA has prohibited the manufacture of some POPs, such as dichlorodiphenyltrichloroethane (DDT), including its derivatives, and Polychlorinated Biphenyls **(**PCBs), specifically PCB-153 (the most ubiquitous non-dioxin like congener), and restricted their usage since 1970s (EPA, 2010). However, endocrine-disrupting compound, such as Bisphenol A (BPA), is still heavily used for variety of reasons around the globe (Global Industry Analysts, 2013).

Bisphenol A:

Bisphenol A [2, 2 bis(4-hydroxyphenyl) propane; BPA] is an endocrine-disrupting compound currently being produced in enormous quantities throughout the world to manufacture polycarbonates in products, such as plastic baby formula bottles, microwavable containers,

dental sealants, plastic toys, inner lining of can products (epoxy resins), and so on. Despite warnings of its deleterious health effects in humans when it percolates into food and beverage (Kharrazian, 2014), more than 10 billion pounds of BPA are produced worldwide on an annual basis, generating an estimated $1 million per day in revenue for plethora of corporations. Startlingly, more than two billion pounds of it are produced in the United Sates (Breast Cancer Fund, 2008).

In the mid-1930s, BPA was used as an estrogen replacement in women due to its ability to bind to estrogen receptors. Later it was found that accumulation of BPA in the body causes adverse health effects in humans, thus the use of BPA as estrogen replacement was halted. Yet, the federal regulatory agencies have failed to recognize the magnitude of BPA's detrimental effects to humans, where even the EPA has considered it to be safe in the 1970s. Over the past 20 years, numerous studies have been conducted implicating it to be not only a pervasive pollutant, but also a potent toxin in the human body (Environmental Working Group, 2008). The effects on human health range from neurological effects, affecting fetal brain development and promoting the development of neurodegenerative disease (Xu et al., 2013), obesity, type 2 diabetes (Taylor, 2013), issues in the digestive system, disruption of hormone production, issues in reproductive system (Nikaido et al., 2004; Howdeshell, Hotchkiss, Thayer, Vandenbergh, Vom Saal, 1999), to involvement in both autoimmune disease development and autoimmune reactivity provocation (Sawai, Anderson, & Walser-Kuntz, 2003 & Shin et al., 2004).

BPA is a single hydrocarbon molecule, as shown on Figure 1., with two phenol rigs connected by a methyl bridge with two methyl groups attached. BPA polymers are formed through a reaction between BPA and carbonyl chlorine in a basic solution. Ester link helps form

polymers with characteristics such as flexibility and long durability, which are highly favored by industries. Heat and hydrolysis due to contact with acidic/basic compounds, lead to breakdown of its ester bonds, releasing BPA into the products consumed, therefore making diet as the primary source of exposure to BPA for most people (Vom Saal & Hughes, 2005).

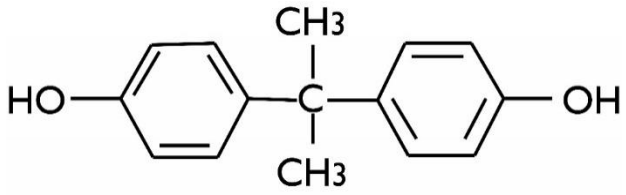


Figure 1. Chemical structure of 2, 2 bis(4-hydroxyphenyl) propane (BPA)

While air, dust, and water are other possible sources of exposure, BPA in food and beverages account for the majority of daily human exposure (European Food Safety Authority [EFSA], 2006). BPA obtained through food chain could accumulate in the body lipid reservoirs of living organisms, and get strenuous as BPA moves from one organism to the other through a process of bioaccumulation (EPA, 2010). Correlated human studies show that according to 2003-2004 National Health and Nutrition Examination Survey, conducted by Center for Disease Control (CDC), detectable levels of BPA were found in nearly 93% of 2517 human urine samples (National Institute of Environmental Health Sciences, 2014), which further emphasizes greater exposure levels of humans to BPA.

Experimental Data about BPA Health Effects:

Neuroendocrine effects: Studies done by Welshons et al show that BPA is an estrogen-mimicking chemical (Welshons et al., 2003). Estrogens are known to have a powerful influence during development that can enduringly alter not only neuroendocrine function, but also the endocrine control of reproductive tracts and mammary glands. When female Sprague-Dawley rats were exposed to 0.1mg/kg body weight/day BPA in the drinking water during gestation and

lactation, disrupted and prolonged estrous cycles and decreased hypersecretion of luteinizing hormone (LH) levels were observed, all of which indicate lasting neuroendocrine effects of early BPA treatment (Rubin, Murray, Damassa, King, & Soto, 2001).

Developmental effects on the female reproductive tracts and mammary glands: Effects of BPA on the reproductive tract can be studied by examining the timing of puberty in female rodents such as the CD-1 mouse (Nikaido et al., 2004). Puberty in female rodents can be assessed by determining the age at vaginal opening or when the first ovulation happens. According to this study method, when exposed to maternal doses between 2.4 and 500 µg/kg/day BPA, early onset of sexual maturation was observed in the female CD-1 mouse strain (Howdeshell et al., 1999). Additionally, developmental effects of mammary glands can be studied through careful observation of the percentage of ducts, terminal ducts, terminal end buds, and alveolar buds of mammary glands during/post exposure to BPA. According to research done by Markey, the stimulation of mammary gland development was observed in female CD-1 mice offspring exposed to low maternal doses of 0.025 and 0.250 µg/kg/day BPA. It is noteworthy that the BPA treatment induced changes in histo-architecture with an increased presence of secretory product within alveoli, resembling early pregnancy (Markey, Luque, DeToro, Sonnenschein, & Soto, 2001).

Similarly, decreased serum testosterone levels were observed in male CD-1 mice exposed to the maternal dose of 2 µg/kg/day BPA (Kawai et al., 2003). In addition, an increase in adult prostate size in male CF-1 offspring was observed when pregnant females were fed BPA at 2 or 20 µg/kg/day (Timms et al., 2005), all of which strongly indicate the developmental effects of

BPA on the male reproductive tracts.

Cancer: Given that BPA is implied to be a primary cause of detrimental health effects in humans (EPA, 2015), a cancer induction due to BPA exposure would not be unprecedented. Numerous studies have been done in this area, relating BPA to various types of cancer development, such as prostate and breast cancer. A study by Murray shows at all levels tested, from 2.5 µg/kg/day to 1000 µg/kg/day, BPA lead to a 3-4 fold increase in the formation of ductal hyperplasia in adult female rats, a form of aberrant cell growth pattern known to be the precursor of breast cancer in both humans and rodents (Murray, Maffini, Ucci, Sonnenschein, & Soto, 2007). By the same token, when NCR/nu/nu mice with implanted human prostate tumor cells were exposed to BPA within the range of common human exposures, it was found that BPA treatment enlarged the tumors significantly compared to the controls, leading to prostate cancer, the most commonly diagnosed cancer among US men (Wetherill et al., 2006).

Type 2 diabetes: In 2006, Alonso-Magdalena et al, studied the effects of BPA on the disruption of pancreatic beta cell function in mice, where the Swiss albino OF1 male mice were injected with 50µg/kg or 5µg/kg BPA. Glycemia levels, insulin secretion, insulin tolerance test, and immunohistochemistry were analyzed. The results indicated that treatment with BPA led to the development of chronic hyperinsulinemia, and altered glucose and insulin tolerance tests. These results suggest that BPA exposure enhances the risk of developing type 2 diabetes mellitus (Alonso-Magdalena, Morimoto, Ripoll, Fuentes, & Nadal, 2006).

BPA and Autoimmune Diseases:

Lupus erythematosus: A low dose of 2.5 µg BPA/kg body weight/day was fed to both normal C57BL/6 and lupus-prone NZB × NZW F1 (NZB/NZW) 5-week-old mice for 1 week.

Analysis of ConA-stimulated splenic mononuclear cells demonstrated that the BPA-fed mice showed a dramatic reduction in IFN-γ and ConA-stimulated interleukin-10 production, indicating, a protective mechanism of BPA in lupus-prone mice (Sawai et al., 2003).

Multiple sclerosis: In efforts to find the enviormental factor that contributes to the increase in the incidence of female multiple sclerosis, Krementsov et al, decided to test the effects of BPA exposure on the development of multiple sclerosis in C57BL/6J and SJL/J mice. Mice were exposed to 10 µg/mL BPA through drinking water and results were analyzed for incidence, cytokine expression, and progression of disease. Startlingly, no significant changes were found in any of the aforementioned parameters compared to controls, indicating that BPA is not a significant contributor to the development of multiple sclerosis in mice (Krementsov et al., 2013).

Type 1 Diabetes and BPA:

There have been two recent studies published suggesting that BPA exposure triggers the onset of experimental T1D (Bodin, Bølling, Samuelsen, et al., 2013; Bodin, Bølling, Becher, et al., 2013). Both studies were performed in NOD mice, one following adult mice exposed to BPA (Bodin, Bølling, Sameulsen, et al., 2013), whereas the second one observed transmaternally BPA-treated mice (Bodin, Bølling, Becher, et al., 2013). According to the 2013 study, when exposed to 1mg/L BPA in drinking water, histological evaluation of female NOD mice has revealed significantly increased insulitis level, and a tendency towards disease acceleration, which did not reach a statistical significance. Study with transmaternal exposure to 10 mg/L BPA showed again more severe insulitis and a tendency towards higher diabetic incidence. Both Bodin's et al studies focused the mechanism of BPA action on the pancreatic islet-residing cells.

Thus, apoptosis of beta cells and tissue residential macrophages was offered as a main explanation of BPA diabetogenic action in NOD females.

However, the crucial evaluation of the immune cell effects due to BPA exposure, particularly T-cells or the cytokines they produce, did not provide a coherent conclusion. Thus, insuficient studies of immune cell functions, such as detection of cytokines in the serum only, were performed in adult NOD mice long-term exposed to BPA, without showing any difference between the controls and BPA treatment groups (Bodin, Bølling, Samuelsen, et al., 2013). A more thorough investigation of immune cell phenotypes, and T-cell functional studies, performed in transmaternally BPA-treated mice, revealed neither changes in proportion of T-cells and their subpopulations, nor differences in proliferative capacity of T-cells and their cytokine secretion, besides a reduction of IL-2 (Bodin, Bølling, Becher, et al., 2013). These insignificant results together suggest that there is a great need for further studies, scrutinizing the effects of BPA on the development of T1D, particularly focused on immune cell parameters.

Effects of BPA on T-cells:

Since it has been known that T1D develops as a result of T-cell dependent destruction of pancreatic beta cells, it is crucial to review what is known about the effects of BPA on the T-cells. (De Maria, 1994). A study by Huimin et al, investigated the effects of adult mouse exposure to BPA on the T-helper immune responses. It revealed a significant promotion of antigen-stimulated production of IL-4 and IL-10, but not IFN-γ, whereas prenatal exposure to BPA showed an increased production of not only IL-4, but also IFN-γ (Huimin, Takamoto, & Sugane, 2008). Overall, the study indicates the development of Th2 cells in mice exposed to BPA during adulthood, and the development of both Th1 and Th2 in the mice prenatally exposed

to BPA (Huimin et al., 2008). Both adulthood and prenatal exposure to BPA decreased the percentage of T-regs, which play a central and prominent role maintaining the immunologic balance (Dieckmann, Plottner, Dotterweich, & Schuler, 2005; Stassen et al., 2004). In another study related to the effects of BPA on T-cell population by Sawai, inhibition of the secretion of IFN- γ in C57BL/6 and NZB/NZW due to BPA exposure was noted (Sawai et al., 2003). In contrast, augmented Th1 immune responses were noted in BALB/c mice exposed to BPA (Alizadeh, Ota, Hosoi, Skai, & Satter, 2006). Similar to the study by Alizadeh, significant up-regulation of Th1 response was observed (compared to control and Th2 response) in male DBA/1 J mice exposed to 3000 μg/kg BPA with increased secretion of IFN- γ. Not only an augmentation in Th1 response, but also enhanced proliferation of splenic lymphocytes in the highest (3000 μg/kg) BPA exposure group, and increased numbers of CD4+ cells and CD8+ cells were observed (Shin et al., 2004). As these contradictory findings about the effects of BPA on T-cells functions in different experimental models were found, further studies are necessary to clarify the underlying mechanism of BPA on the development of T1D in the context of its influence on T-cells.

Chapter II- AIM

Concerning the relationship between POPs and T1D in NOD mice, recent studies reported aggravation of disease with exposure to DDE (Cetkovic-Cvrlje et al., 2015), and suppression of T1D with PCB-153 (Kuiper et al., 2016). Considering BPA association with T1D, a potentiation of disease was suggested by Bodin et al, primarily through BPA effects on insulitis development; however, a significant difference in the incidence of T1D in BPA-exposed NOD mice was not confirmed. Whereas it has been well documented that BPA affects T-cells and their function, BPA effects on the immune system in general, and T-cells in particular, during the development of T1D were not clarified (Bodin, Bølling, Samuelsen, et al., 2013).

Therefore, the aim of this study was to investigate effects of BPA exposure on T1D development in a MLDSTZ mouse model. As T1D is a T-cell-dependent and -mediated disease (Cetkovic-Cvrlje et al., 2003 & 2012), the effects of BPA on T-cell function were studied both *in vitro* and *in vivo* in order to address potential mechanisms of BPA action. It was hypothesized that BPA would affect the incidence of T1D in MLDSTZ-treated mice through alteration of T-cell composition/function. To test this hypothesis, glucose and insulitis levels, along with spleen cell counts, viability, T-cell proliferation, T-cell subsets (and other immune cell types), and cytokine levels were determined during exposures of MLDSTZ-treated C57BL/6 mice to BPA.

Chapter III- MICE AND METHODS

Mice:

C57BL/6J mice were purchased from the Jackson laboratory (Bar Harbor, ME, USA) and bred in St. Cloud State University vivarium (SCSU). All procedures performed on the mice were approved by the SCSU Animal Care and Use Committee (protocol # 5-75) before the start of the project. BPA free cages were used for animal housing and mice were allowed for free access to water and food with casein-based phytoestrogen-free diet (AIN-93G Rodent Diets, Research Diet, Inc., Harlan research laboratories). These mice were exposed to a 12-hour light/dark cycle and were kept in separate NexGen Lo-Profile 70-cage BPA-free cages (Allentown, Inc., Allentown, NJ) based on sex, while the male mice were chosen for experiment based on age. Four-weeks-old male C57BL/6 were used for the experiments throughout the course of this project. Mice were observed both before and throughout the entire experimental period to ensure the absence of health issues or physical infirmities. During the experimental endpoints and/or upon detection of detrimental heath issues animals were euthanized using CO_2 asphyxiation.

Experimental Design:

In vivo experiments: In order to study the effects of BPA *in vivo,* four weeks old male C57BL/6J mice were divided into two groups: control and treatment. Treatment groups received 1mg/L or 10mg/L BPA dissolved in deionized (DI) autoclaved drinking water, and were followed up to 16 weeks of age. Control mice received DI autoclaved drinking water without BPA. At the age of 9 weeks, autoimmune diabetes was induced in both control and BPA-treated group of mice by five low doses of STZ (MLDSTZ model). Twice a week, glucose measurements were taken from day eight and followed up to day 50 post first STZ injection.

Pancreata and spleens were harvested on days 11 and 50 post first STZ injection in order to study histopathology (insulitis), T-cell and other immune cell type composition (B-cells, macrophages, and natural killers), and function of T-cells (T- cell proliferation assay and cytokine detection) during the development of T1D, as shown in Figure 2.

BPA preparation and solution intake: 1 and 10 mg solid BPA were measured using a standardized scale. BPA was dissolved in 0.01v/v% ethanol (BPA has high solubility in alkaline solutions); then mixed in 1L deionized (DI) autoclaved drinking water, and heated up to 60 ◦C (Thien, 2009) for the preparation of both BPA concentrations 1 and 10 mg/L respectively. For controls, ethanol was added to the autoclaved DI water in the same concentration as present in the BPA treatment solution, to ensure no differences observed were from the ethanol addition. As per the literature (Bodin, Bølling, Samuelsen, et al., 2013), an addition of BPA to drinking water in the concentrations proposed in this protocol (1 and 10 mg/L), did not lethally affect animals or their drinking habits and water intake. According to our pilot studies, an average non-pregnant mouse of 25 g drinks about four ml water/day, regardless of the BPA addition. Based on water intake measurement, the amount of BPA a mouse got was 160 µg/kg (1 mg/L) and 1600 µg/kg (10 mg/L) body weight. The water was administered in BPA-free glass bottles and levels were checked daily and refilled accordingly. In addition, all the water bottles were autoclaved and changed weekly with fresh BPA solution to ensure the intake of uncontaminated water solution by mice.

Induction of MLDSTZ-induced T1D in C57BL/6 mice**:** STZ was administered to both control and BPA-treated C57BL/6 mice in five consecutive intraperitoneal injections in a dose of 40mg/kg based on their body weight. Appropriate amount of STZ/day was measured in

autoclaved 1.5 mL Eppendorf tube based on the average weight of all the mice and the total number of mice of that group. The Eppendorf tubes were sealed tightly to avoid any loss of STZ due to air contact (STZ is an aerosol compound). Right before the injections, the tube of STZ was dissolved in 0.05M sodium citrate buffer made with cold DI water. The buffer pH was set to 4.5 with appropriate volume of 1M HCl and NaOH. The solution was then vortexed at high speed to create a drug suspension and were injected in a volume of 6.7 µL/g of body weight (Ter Veld et al., 2008) within 20 minutes to avoid drug precipitation. Animal activity was monitored right after injection for any abnormal signs. The basic formula to prepare 0.05M sodium citrate buffer is as follows:

Molarity of sodium citrate (0.05M) x Molar mass of sodium citrate = weight of sodium citrate solid (grams).

Desired amount of sodium citrate solid was measured based on the number of mice injected and dissolved in the appropriate volume of cold DI water to prepare 0.05M buffer. The basic formula to prepare 40 mg/kg STZ is as follows:

$$\frac{40\ \text{mg} \times \text{Average mouse weight (g)}}{1000\ \text{g body weight}} = \text{Amount of STZ to be used to inject one mouse}$$

Based on the above formula, appropriate amount of STZ was measured for the total number of mice injected on a particular time and dissolved in required volume of sodium citrate buffer. The basic formula for the appropriate volume of injection buffer is as follows:

6.7 µL/g of body weight x Average mouse weight of the group to be injected (g) x Total number of mice injected = Required volume of buffer/day.

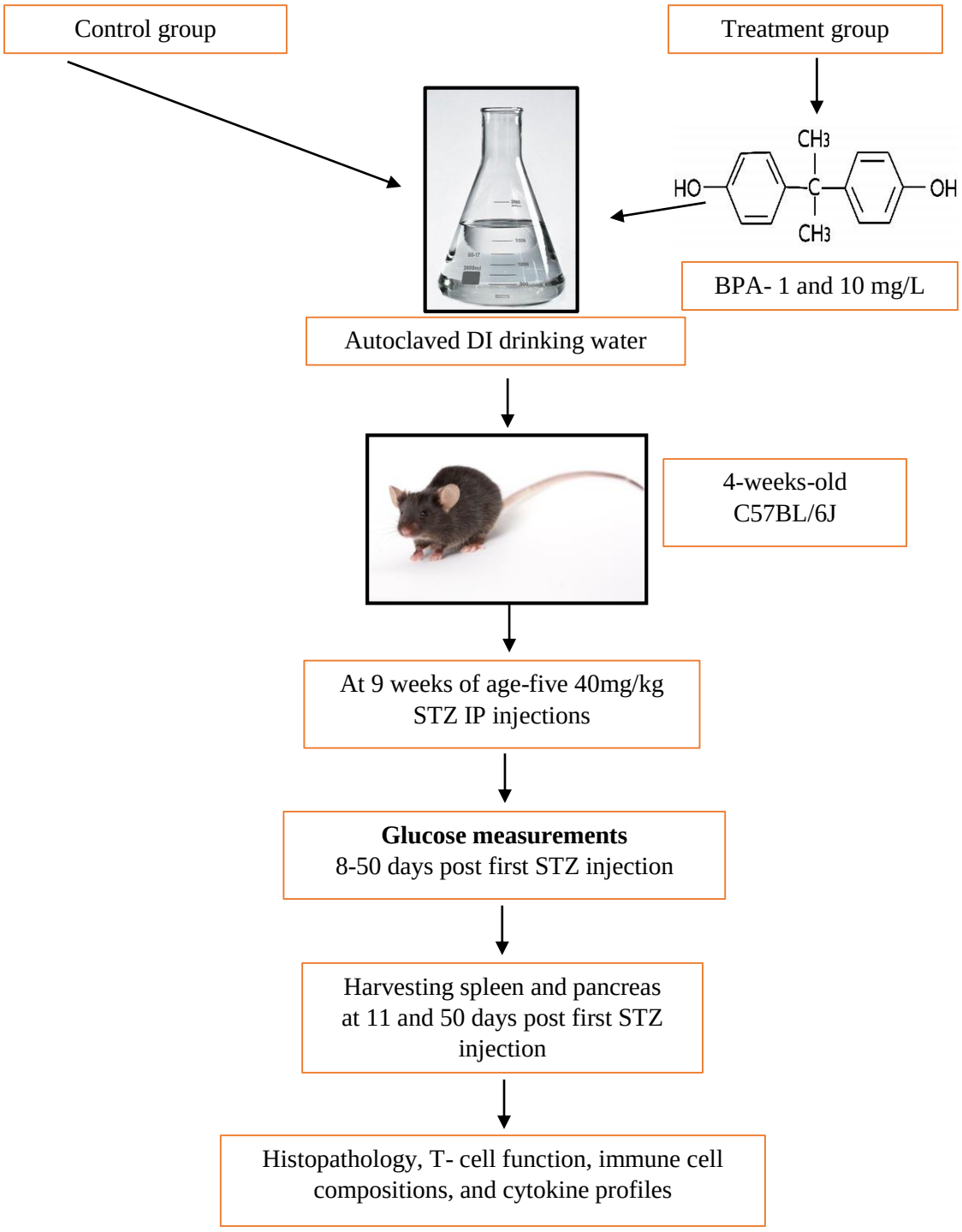


Figure 2. Flow chart representing the experimental design of BPA experiments in MLDSTZ-induced T1D in C57BL/6J mice.

Diabetes Incidence and Onset Determination:

Incidence of T1D in MLDSTZ-treated and BPA-exposed C57BL/6J mice was monitored by determination of hyperglycemia from eighth day until 50th day post first STZ injection. To determine the onset of diabetes, weight and the blood glucose levels were measured simultaneously. To check the blood glucose level, blood was obtained from lateral tail vein and analyzed by Accu-Check glucose monitor. For blood glucose measurements from the tail vein, mice were restrained (placed in a 50 ml Falcon tube with breathing hole and a hole in the cap for the tail), and the tail was kept for 15 seconds in a 37°C water bath and massaged to induce vasodilation of tail blood vessels. A drop of blood was obtained by venipuncture (0.6 µl of blood is needed for a single measurement), and placed on Accu-Check glucose strips (Roche Diagnostics, Indianapolis, IN, USA). The lateral tail vein blood sampling procedure is considered to have minimal physiological impact on the animal and requires no anesthesia, allowing for an accurate measurement of blood glucose **(**Massachusetts Institute of Technology, 2016)**.** Mice were considered diabetic after two consecutive readings of blood glucose levels of 220 mg/dL or more, for 2 consecutive measurements.

Determination of Insulitis:

Diabetes occurrence is associated with a development of typical histopathological lesion of the pancreatic islets of Langerhans, called insulitis. Insulitis represents the mononuclear cells infiltration of islets, and its level is believed to be associated with the level of beta cell destruction. In this study, mice were euthanized and pancreata harvested on day 50 post first STZ injection, fixed using 10% formalin solution, and embedded in paraffin wax. Then pancreata were cut to 5µm sections in triplicates using microtome, and stained with hematoxylin and eosin.

Stained tissues were observed under light microscope to determine insulitis levels, which were semi-quantified by determining insulitis index. The insulitis index was determined by grading the islets on a scale from zero to four, based on the level of mononuclear cell infiltration into the islets of Langerhans. The representation of the grading scale is as follow: level 0 - no infiltration, level 1- less than 25% intraislet infiltration, level 2 - >25-50% intraislet infiltration, level 3 - >50-75% intraislet infiltration, and level 4- more than 75% infiltration. The averages of insulitis index were then compared between control and BPA-treated groups. The formula used to calculate insulitis index is given below:

$$\textit{Insulitis index} = \frac{(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)}{\textit{Total number of islets}}$$

In the above formula, n_0, n_1, n_2, n_3, and n_4 represent the number of islets scored at grades 0, 1, 2, 3, and 4 respectively.

***In Vitro* Experiments:**

To determine the effects of BPA on T-cell function *in vitro*, splenocytes of untreated non-diabetic C57BL/6J mice were obtained. Single cell suspensions were made to acquire splenocytes. After determining cell counts and viability, cells were stimulated by a mitogen concanavalin A (ConA, Sigma, St. Louis, MO, USA) in the concentration of 3 µg/mL in complete media (RPMI- 1640 enriched with 1 unit/mL Penicillin and Streptomycin and 10% fetal calf serum; Sigma, St. Louis, MO, USA). A serial dilution of BPA was prepared from 40 mg/mL stock and then diluted in complete media to create a range of concentrations from 1.56 to 100 µg/mL. Appropriate doses of BPA were then added to corresponding wells with either splenocytes in medium or splenocytes in ConA. In addition, a baseline control of just cells and medium (for unstimulated) or ConA (for stimulated) cells were used to compare T-cell

proliferation under control conditions. Cells were cultured in 96-well-plate in the concentration of $4x10^6$ cells/mL in triplicates with the addition of adequate amount of BPA and ConA in a total volume of 100 µL. Splenocytes were incubated in a growth chamber at 37ºC and 5% CO_2 for 72 hours. T-cell proliferation was then analyzed by use of Alamar Blue (Invitrogen, Grand Island, NY, USA) colorimetric assay according to the manufacturer's instructions, and absorbance read by spectrophotometer (Elisa reader; GeneMate, Kaysville, UT, USA) at 570nm.

Apoptosis Detection:

BPA has been known to induce apoptosis of different cell types (Bodin, Bølling, Samuelsen, et al, 2013; Lin et al, 2012 & O'Brien, Harmon, Cameron, and Allan, 1996). Apoptosis analysis was performed by the use of FITC Annexin-V Apoptosis Kit; BD Pharmingen, San Diego, CA, USA. Splenocytes were cultured in the concentration of $4x10^6$ cells/mL with the addition of either 1.56, 6.25, or 12.5 µg/mL BPA in a 12- well- plate. As for control, splenocytes were cultured with complete media without any addition of BPA. Cultures were incubated in a growth chamber at 37ºC and 5% CO_2 for 24 hours. After incubation, splenocytes were collected, washed with 1X PBS, and $4x10^5$ cells from each sample were suspended in 100 µL binding buffer according to the instruction manual. In order to analyze apoptotic cell events, splenocytes were incubated for 15 minutes at room temperature, protected from light with the addition of 5 µL of reagents. The samples were then filled with adequate amount of binding buffer up to a total volume of 400 µL. BD FACSCalibur flow cytometer (BD Biosciences) was used to analyze the samples. In this apoptosis assay, apoptotic cells display protein phosphatidylserine (PS) on its cell surface, which binds strongly to protein Annexin-V. The protein Annexin-V is conjugated with fluorochrome FITC which allows PS^+ cells to be

detected by flow cytometry. In addition, another fluorochrome, propidium iodide (PI), is used to distinguish between apoptotic and necrotic cells. PI enters into dead cells through cell membrane and binds to its nucleic acids, whereas alive cells do not allow uptake of PI. FITC single positive events represent early apoptotic cells, FITC/PI double positive events represent late apoptotic cells, and PI single positive cells represent necrotic cells in flow cytometer output.

Immune Parameters:

The investigation of immune cell parameters were done on days 11 or 50 post first STZ injection in both control and BPA-treated group of mice to determine the effects of BPA exposure on the immune cell population. These parameters include splenic cell count and viability, T-cell proliferation, characterization of immune cell populations/subpopulations through immunophenotyping process, and cytokine profiling.

Single cell suspension, cell counts and viability determination: Immune cells can be subjected to different immunological analysis by an initial laboratory technique called preparation of single cell suspension. At the predetermined end points, or when mice became diabetic, they were euthanized and spleens were harvested aseptically for the preparation of single cell suspensions. Spleens were placed onto separate 40µm nylon strainers (BD Falcon, USA) with one mL of 1X PBS buffer and placed on top of 50mL conical tubes. Using a plunger from a 10mL sterile syringe, the spleens were meshed and repeatedly washed with 1X PBS to acquire a total volume of 5 mL spleen suspension in the conical tube. Then, the suspension from each spleen sample were transferred to separate labeled 15mL conical tube, and centrifuged at 1200 rpm for five minutes (Beckman CS-6R centrifuge). After the centrifugation, the supernatant was decanted into a waste container, and cell pellet was resuspended with 750 uL ACK lysis

buffer (NH_4Cl 8.29 g/L, $KHCO_3$ 1.0 g/L, EDTA $Na_2 \cdot 2H_2O$ 0.0375 g/L; Lonza BioWhittaker, Walkersville, MD, USA) for one minute at the room temperature to lyse the red blood cells. The cells were then suspended with 5 mL of 1X PBS and centrifuged. Three more washes were done to remove all the remaining lysis buffer and cell debris from the suspensions. Lastly, cell counts and viability were determined through Trypan Blue (Lonza BioWhittaker, Walkersville, MD, USA) exclusion method. Splenocytes were mixed with Trypan blue in the ratio of 1:20, and placed onto a hemocytometer. Hemocytometer was then placed under 40X magnification in a light microscope to count both alive and dead splenic cells. Trypan blue stained the dead cells to appear blue in color while allowed the alive cells to appear bright and unstained.

T-cell proliferation assay: To determine the effects of *in vivo* BPA treatment on T-cells, functionality of T-cells was analyzed through T-cell proliferation assay. Mice were euthanized after BPA treatment *in vivo* at predetermined endpoints and spleens were harvested aseptically in order to obtain the single cell suspensions. After the single cell suspensions were obtained, splenocytes were used for culturing based on their viability. Splenocytes were cultured in complete media for 72 hours in the concentration of $4x10^6$/mL in a total volume of 100 µL in a 96-well-plate with addition of 3 µg/mL Con A to stimulated wells or complete media to unstimulated wells. After 72 hours of incubation of splenic cells in a growth chamber with/without Con A (37ºC and 5% CO_2), Alamar blue colorimetric assay was performed. 10 µL of Alamar blue (10% of the total volume) was added to the proliferation wells and incubated for an additional five to eight hours. Alamar Blue is a redox indicator that is used to evaluate metabolic function and cellular health. The assay reduces the active component resazurin which appears blue in color, to resafurin, which appears red-fluorescent, in well-proliferated splenocyte

samples (Rampresad, 2012). After five to eight hours of incubation in the aforementioned growth chamber, culture plates were read spectrophotometrically at 570 nm to quantify optical density of cultured T-cell supernatants. Well-proliferated stimulated culture wells appeared bright pink in color, representing increased function of T-cells, while the non-stimulated wells and controls (complete media only) appeared blue.

Immunophenotyping Study:

The alterations in the composition of T-cell types and sub types, B-cell, macrophages, and NK cells during and post BPA exposure were studied by flow cytometric immunophenotyping analysis. After single cell suspensions are made, splenocytes were used for immunophenotyping in a concentration of $1x10^6$/mL. Appropriate amount of splenocytes were delivered into FACS polystyrene tubes with 1 mL of FACS buffer (0.1% NaN_3, 1% Fetal Calf Serum, and PBS) and centrifuged at 1200 rpm for five minutes. Supernatant was decanted and tubes were blotted to remove any leftover buffer. Splenocytes then were labeled with antibodies conjugated with fluorochromes against particular proteins they express on their cell surface, called clusters of differentiation (CD). CDs represent different types of immune cells, which are as follow; CD4, CD8, CD4 and CD25, CD45R/B220, NK1.1, CD11b, and CD3 which define Th, Tc, Tregs, B-cells, natural killer cells, macrophages, and total T-cells, respectively. The following were the fluorochromes by which the antibodies were conjugated; Fluroscein isothiocyanate (FITC), Phycoerythrin (PE), Peridinin chlorophyll (PerCP), and Allophycocyanin (APC). The antibodies that were used to detect the aforementioned cell surface markers, include: anti-CD4 PerCP (clone RM4-5), anti-CD8 FITC (clone 53-6.7), anti-CD25 APC (clone 3C7), anti-B220 APC (clone RA3-6B2), anti-NK 1.1 FITC (clone PK 136), anti-CD 11b PerCP (clone

M1/70), and anti-CD3 PE (clone 145-2C11) (all from BD Biosciences).

Antibodies were prepared in 1:100 ratio with FACS buffer. After the addition of antibodies, cell were incubated for 30-45 minutes at 4◦C, protected from light, allowing the antibodies to bind to appropriate cell epitopes. Post incubation, cells were washed three times with 500 µL of cold FACS buffer solution to remove any unattached antibodies. After the final wash, cells were resuspended in 300 µL of cold FACS buffer. To quantify individual immune cell type, labeled splenocytes were run through flow cytometer. Flow cytometer allows for the identification of the heterogeneity of the immune cells which can be achieved through the analysis of multiple surface and intracellular markers (Lugli et al., 2011). As the samples run through the flow cytometer, it excites laser towards the labeled splenocytes with fluorochrome attached antibodies. Each fluorochrome then emits a photon of light with different wavelengths that correspond to five different channels which can distinguish cells from one another and describe cell characteristics. The first channel is fluorochrome-independent side scatter and forward scatter, which distinguish cells based on their size and granularity, respectively. The next four channels are fluorochrome-dependent and appear as FL1, FL2, FL3, and FL4. They require fluorochromes conjugated with antibodies to differentiate cell types. The corresponding fluorochromes for these channels are as follow; FL1 detects FITC, FL2- PE, FL3- PerCP, and FL4- APC. Following acquisition of antibody-labeled cells, the composition of the immune cells of interest was analyzed by CellQuest Pro software.

Cytokine Collection and Analysis:

To detect cytokine levels in the supernatants of the stimulated splenocytes, spleen were obtained from the experimental mice at predetermined endpoints. After obtaining single cell

suspension, splenocytes were added to a 24-well plate in the volume of 500 µl to achieve a final concentration of $4x10^6$/mL, with addition of 3 µg/mL Con A for the stimulated wells, and complete media for the unstimulated wells. Cells were incubated for 48 hours inside a growth chamber at 37ºC and 5% CO_2. After the incubation, cells were centrifuged at 1200 rpm for 10 minutes. The cell supernatants, that contain cytokines, were then removed gently in two 250 µL aliquots and freezed at -80ºC for cytokine analysis. Cytokine quantification was done by using the flow cytometer and cytometry-based bead assay (BD™ CBA Mouse Th1/Th2/Th17 Cytokine Kit; BD Biosciences). As the initial step, lyophilized stop standard solution was prepared with 2 mL of assay diluent provided by the manufacturer. A serial dilution was then done in a ratio of 1:2 until the final dilution was reached 1:126. Next, the total capture bead mixture was prepared by adding 10 µL of all the beads to one tube. These beads are specific for certain cytokines of interest. The capture bead mixture was then added to each serial diluted tubes in an amount of 50 µL, followed by the addition of 50 µL thawed cell supernatant. As the final step of this process, 50 µL of fluorochrome PE, the detection agent, was added to each tube and incubated for two hours at 4ºC, protected from light. Samples were then acquired using flow cytometer and analyzed using FCAP Array software (Softflow, New Brighton, MN, USA) to detect the following cytokines: IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ, and TNF-α.

Statistical Analysis:

The differences in diabetes incidence of control and BPA-treated group of mice were analyzed by the life-table statistical method and log rank test (Mantel-Cox), using the statistical software program SPSS (IBM, Armonk, NY, USA). The statistical differences of glycemia levels and body weights between the control and BPA- treated groups were determined through one-

way ANOVA with repeated measures. The differences in insulitis index, in vitro proliferation, apoptosis, stimulated/non-stimulated T-cell proliferation, immune cell composition, and cytokine secretion between control and BPA-treated groups were performed using Excel, two-tailed, unpaired student's t-test. $p<0.05$ was considered as statistically significant for all performed statistical analyses.

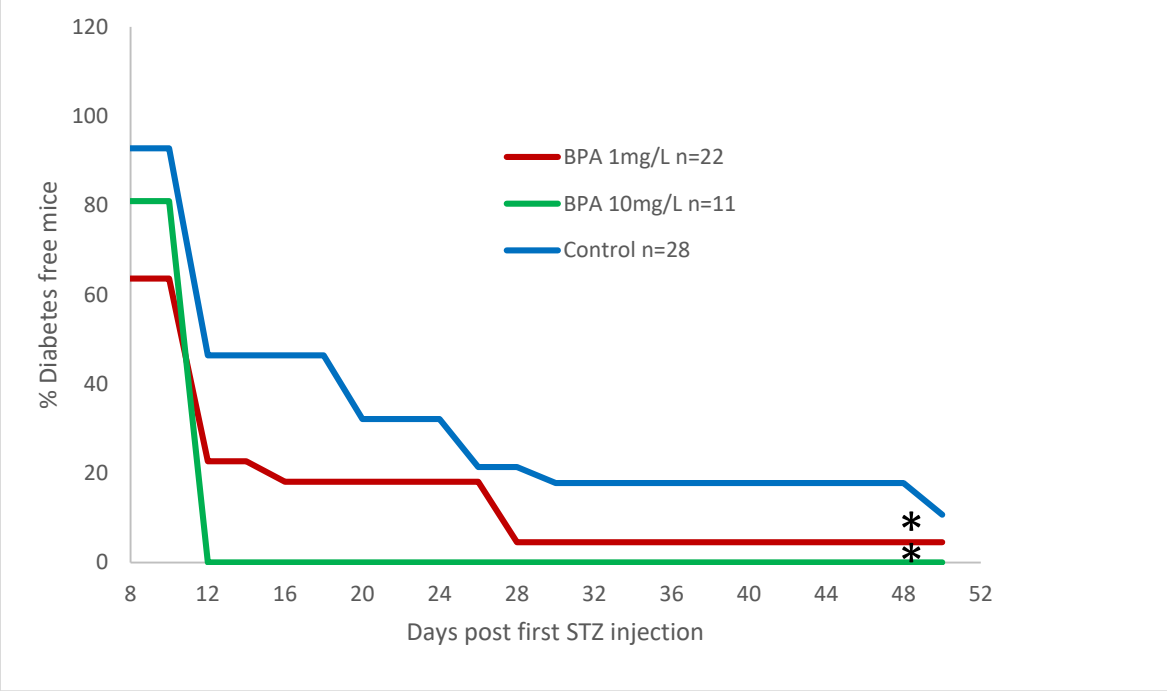
Chapter IV- RESULTS

Effects of Chronic BPA Exposure on TID Incidence and Glycemia Levels in MLDSTZ Mouse Model of T1D

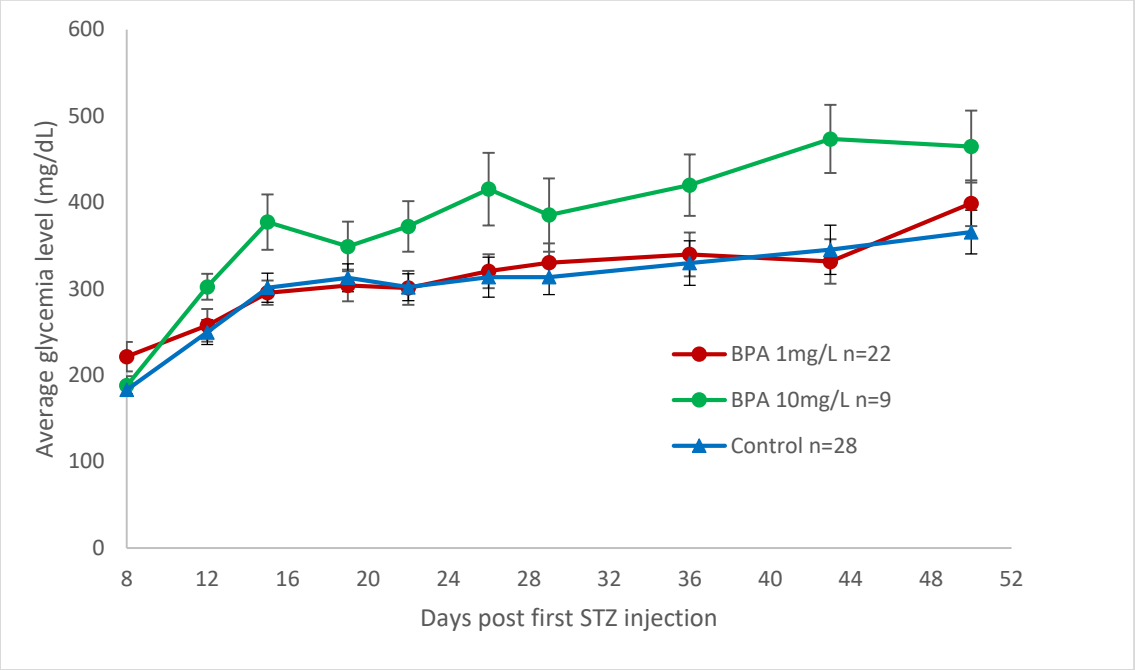
Non-diabetic, healthy, four-weeks-old C57BL/6J mice were exposed to low- (1 mg/L), high (10 mg/L)-doses of BPA, and vehicle control treatments until they reached 16 weeks of age. At nine weeks of age, mice received 40 mg/kg body weight multiple low-doses of streptozotocin (MLDSTZ) injections intraperitoneally for 5 consecutive days. Glucose measurements and body weights were taken biweekly from the eighth day post the first STZ injection until the experimental endpoint, 50 days post the first STZ injection (16 weeks of age). There were no significant differences at the beginning of the experimental period in glucose measurements (156.7±8.8, 169.9±7.5, and 177.7±5.7 mg/dL in control, low-dose and high-dose group, respectively) or body weights (data not shown) between groups. During the course of the 12-week treatment period, body weight measurements did not differ among any of the aforementioned experimental groups, indicating no signs of direct toxicity (Figure 3C). In addition, no clinical signs of any detrimental health effects were observed. The overall incidence of T1D significantly increased in both low- and high-doses BPA-exposed groups compared to controls, with the high-dose being the most significant ($p=4.1x10^{-2}$ and $p=1.6x10^{-2}$, respectively) (Figure 3A). At the first day of glucose measurement (8^{th} day post the first STZ injection), 36.4% of the mice from the high-dose BPA-exposed group and 19% from the low-dose BPA-exposed group became diabetic, compared to only 7.2% in control group. At the 12^{th} day post the first STZ injection, 100% of the mice from high-dose BPA-exposed group became diabetic, in comparison to 77.3% in the low-dose group and 53.6% of the controls; 95.5% of low-dose

exposed mice became diabetic on day 29 and 89.3% of controls on day 50 post first STZ injection. In addition, the overall glycemia levels showed increased trend in the high-dose BPA exposed group compared to the controls throughout the experiment (Figure 3B). However, statistically significant differences were not observed. Overall, *in vivo* studies of incidence, glycemia and insulitis levels suggest that both low- and high-doses of BPA, accelerate the development of disease in MLDSTZ C57BL/6J T1D model.

A.



B.


600
500
400
300
200
100
0
Average glycemia level (mg/dL)
8
12
16
20
24
28
32
36
40
44
48
52
Days post first STZ injection
BPA 1mg/L n=22
BPA 10mg/L n=9
Control n=28

C.

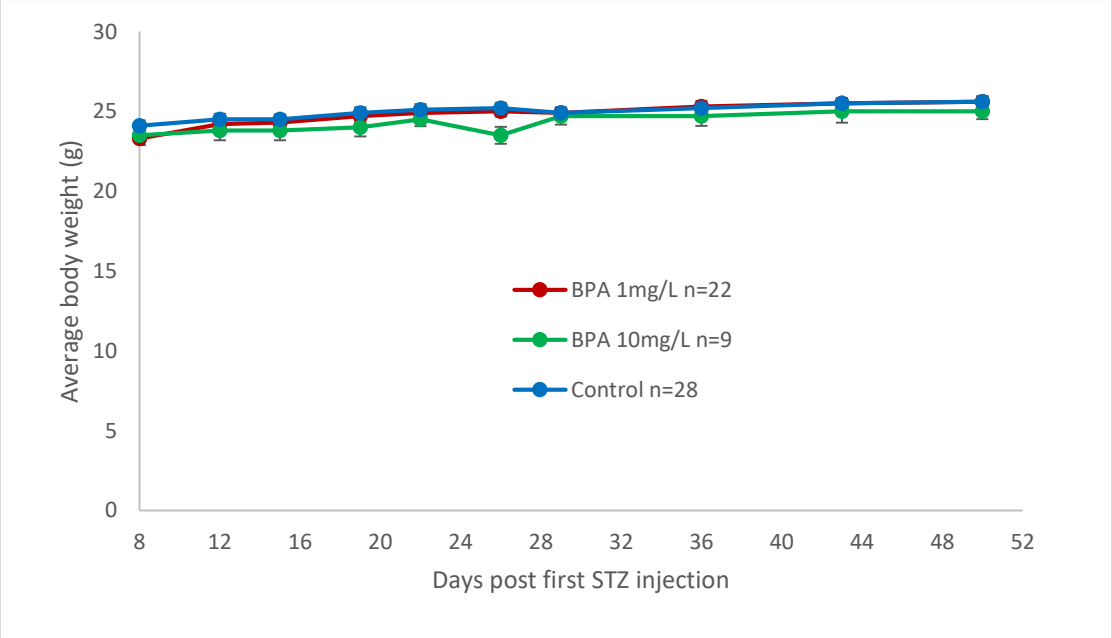

30
25
20
15
10
5
0
Average body weight (g)
8
12
16
20
24
28
32
36
40
44
48
52
Days post first STZ injection
BPA 1mg/L n=22
BPA 10mg/L n=9
Control n=28

Figure 3. Diabetes incidence (A), average glycemia (B), and average body weights (C) in MLDSTZ-treated C57BL/6J male mice exposed to BPA for 12 weeks. Mice were exposed to either 1 mg/L, 10 mg//L BPA, or control buffer dissolved in drinking water from four to 16 weeks of age. (A) Diabetes incidence presented as percent diabetes free mice; significance determined by Mantel-Cox log rank test (*$p \leq 0.05$ compared to control). (B) Glucose measurements were taken after MLDSTZ injections starting from eighth day up to 50 days post first STZ injection. A mouse with two consecutive measurements of glycemia with 220 mg/dL or more was considered diabetic. Data are shown as mean ± SEM. (C) Body weights were measured (g) prior to each glucose measurement up to the experimental endpoint; data are presented as mean ±SEM.

Effects of BPA Exposure on the Insulitis Level of Pancreatic Islets in MLDSTZ-treated C57BL/6J Male Mice

Showing the clear association between BPA and accelerated T1D incidence, the insulitis level was analyzed in order to semi-quantify mononuclear infiltration of pancreatic islets in MLDSTZ-treated C57BL/6J mice exposed to BPA. Mice were treated with STZ and BPA, as described for the diabetes incidence studies, euthanized, and pancreata harvested at 16 weeks of age (experimental end point). Insulitis index was calculated for each mouse. Results revealed a significant increase in the average insulitis index in the low- and high-dose BPA-exposed groups compared to the control ($p=2.9x10^{-5}$ and $p=7.9x10^{-3}$, respectively) (Figure 4), indicating increased infiltration of inflammatory cells into the pancreatic islets after chronic exposure to BPA. Both groups of BPA-exposed mice exhibited similar insulitis levels.

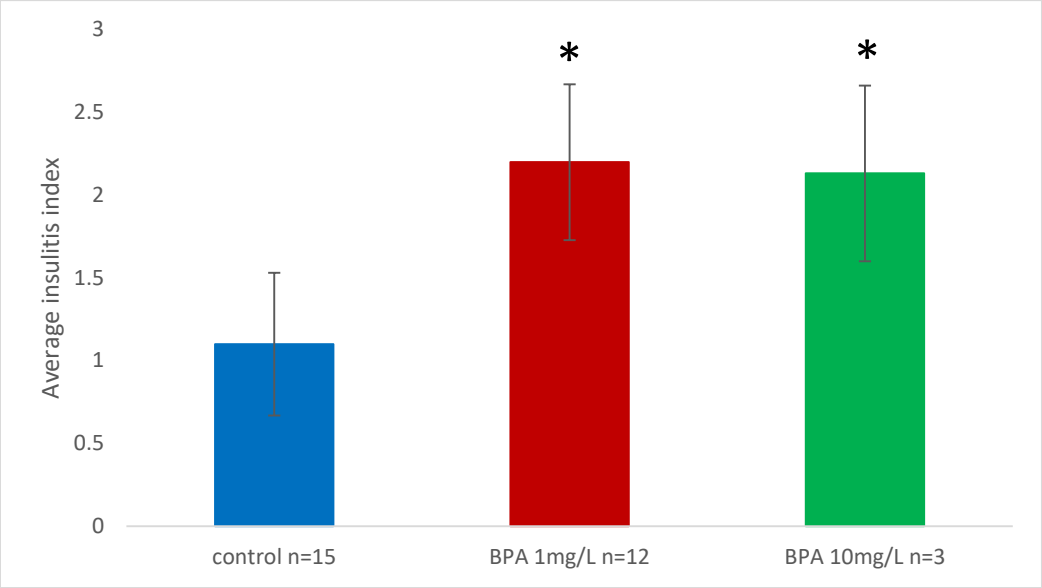


Figure 4. Average insulitis index in MLDSTZ-treated C57BL/6J male mice exposed to BPA for 12 weeks. Mice were exposed to either control, 1 mg/L, or 10 mg//L BPA dissolved in drinking water from four to 16 weeks of age. Mononuclear cell infiltration of pancreatic islets was graded as 0, 1, 2, 3, and 4, based on the severity of infiltration, and insulitis index was calculated. Data are shown as mean ± SEM; t-test (*$p \leq 0.05$ compared to controls).

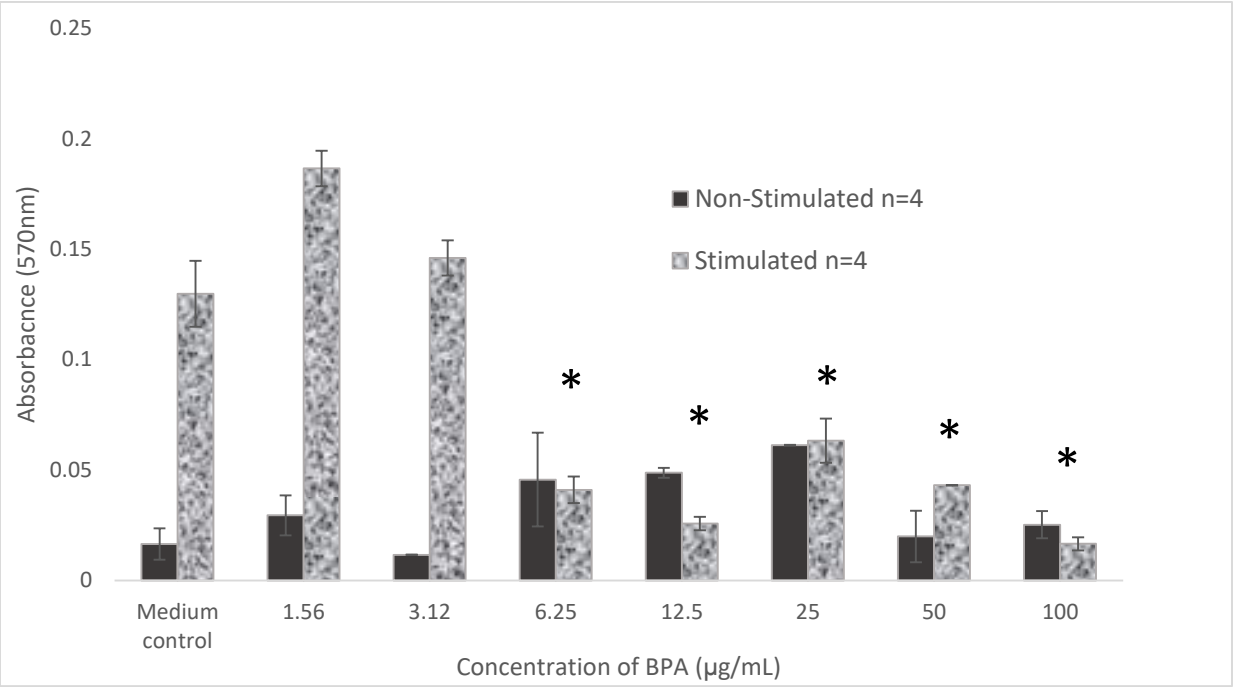
***In Vitro* Effects of BPA on T-cell Function**

Since T1D is a T-cell-mediated disease, it was asked whether BPA could directly affect T-cell function, by studying T- cell proliferation induced in C57BL/6J splenocytes by T-cell mitogen ConA (3µg/mL) *in vitro.* A serial dilution of BPA/PBS was made and added to a 96-well plate plated with splenocytes, ranging from 1.56 µg/mL to 100 µg/mL. ConA-induced splenocyte proliferation in culture medium without any addition of BPA was used as a control. It was determined that 0, 1.56 and 3.12 µg/mL BPA did not reduce ConA-induced T-cell proliferation, whereas higher doses, 6.25, 12.5, 25, 50, and 100 µg/mL BPA suppressed the proliferation ($p=8.0\times10^{-3}$, $p=2.0\times10^{-3}$, $p=1.3\times10^{-2}$, $p=5.0\times10^{-2}$, and $p=7.7\times10^{-3}$, respectively), compared to medium only-exposed controls (Figure 5A).

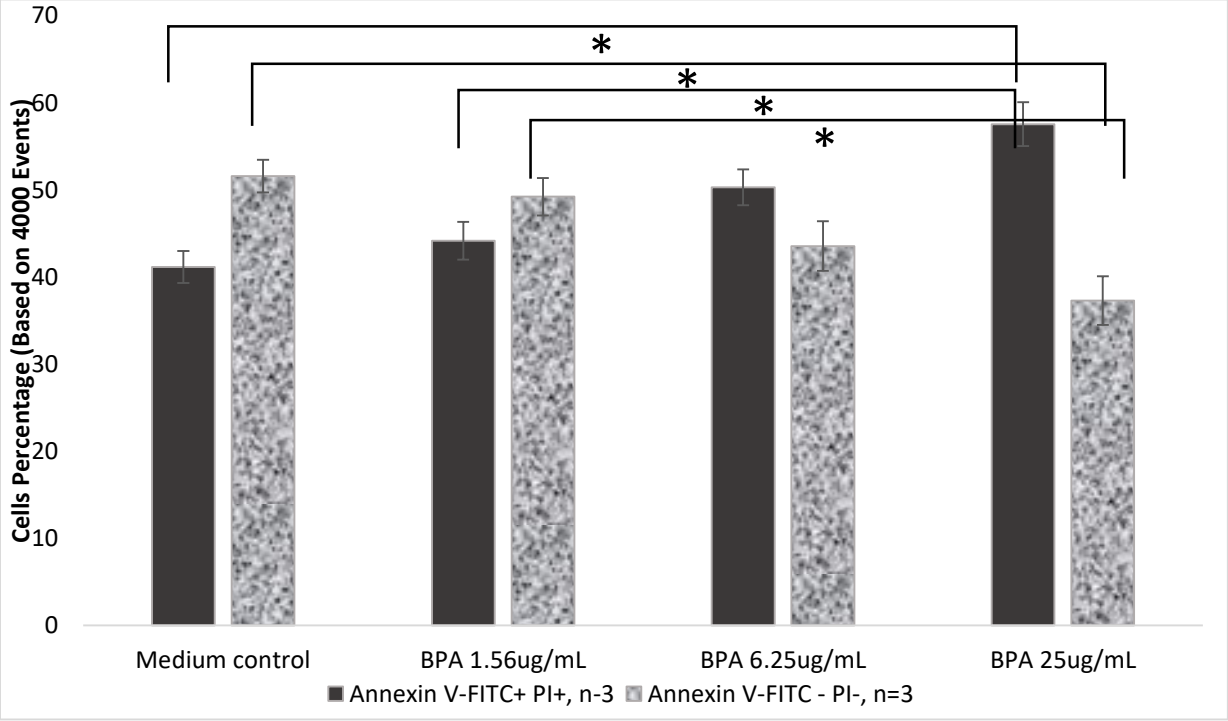
Plethora of research has been done indicating that BPA induces apoptosis of different cell types

(Bodin, Bølling, Becher, et al, 2013; Lin et al, 2012). In order to determine if the reduced proliferation in BPA-exposed cultures of T-cells were caused by apoptotic induction, Annexin-V flow cytometric-based assay was performed. Splenocytes of untreated C57BL/6J male mice were incubated for 24 hours with addition of 0, 1.56, 6.25, and 25 µg/mL BPA. These BPA concentrations were chosen based on proliferation assay as a representative concentration range that were shown to be able to reduce (6.25 and 25 µg/mL), or not reduce (1.56 µg/mL) the proliferation of T-cells (Figure 5A). Annexin-V FITC/PI staining revealed that addition of 25 µg/mL BPA significantly changed the percentage of alive/dead cells compared to 1.56 µg/mL, as well as to medium control (Figure 5B). Alive cells (Annexin-V^- $FITC^-$) were significantly reduced, while the apoptotic cells (Annexin-V^+ $FITC^+$) significantly increased in the cultures exposed to 25 µg/mL BPA compared to 1.56 µg/mL and the control ($p=2x10^{-3}$ and $p=1.0x10^{-3}$; $p=2x10^{-3}$ and $p=1.0x10^{-3}$, respectively) (Figure 5B and 5C). Cells exposed to 6.25 µg/mL BPA, while showing increased percentage of apoptotic and decreased percentage of alive cells, did not reach significant difference compared to control. Data obtained from *in vitro* studies suggest that higher concentrations of BPA has the ability to inhibit T-cell proliferation, possibly by inducing apoptotic cell death.

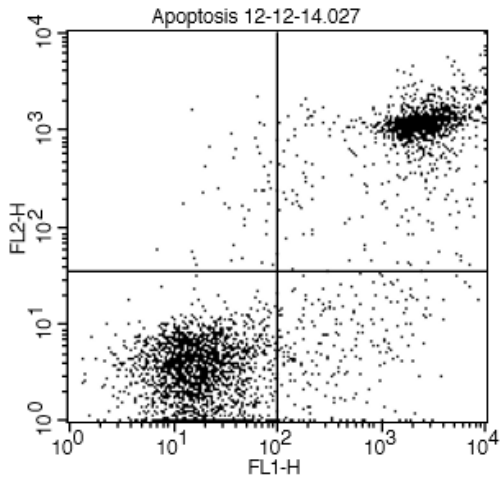
A.


0.25
0.2
0.15
0.1
0.05
0
Absorbacnce (570nm)
Non-Stimulated n=4
Stimulated n=4
*
*
*
*
*
Medium control
1.56
3.12
6.25
12.5
25
50
100
Concentration of BPA (µg/mL)

B.


70
60
50
40
30
20
10
0
Cells Percentage (Based on 4000 Events)
*
*
*
*
Medium control
BPA 1.56ug/mL
BPA 6.25ug/mL
BPA 25ug/mL
Annexin V-FITC+ PI+, n-3
Annexin V-FITC - PI-, n=3

C.

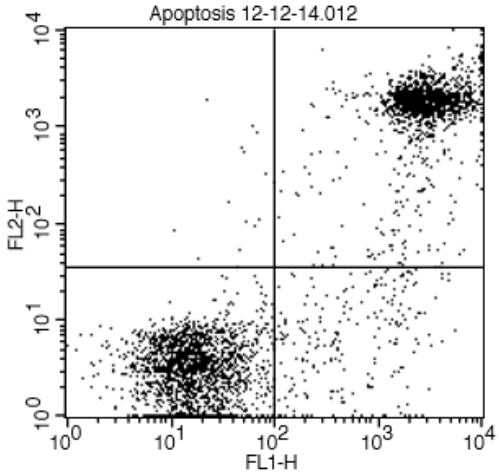


Quadrant Statistics

File: Apoptosis 12-12-14.027
Patient ID:
Panel: Apoptosis JK
Gate: No Gate
Total Events: 4020

Sample ID:
Tube: #28 Cells
Acquisition Date: 12-Dec-14
Gated Events: 4020

Quad	Events	% Gated	% Total
UL	42	1.04	1.04
UR	1506	37.46	37.46
LL	2238	55.67	55.67
LR	234	5.82	5.82



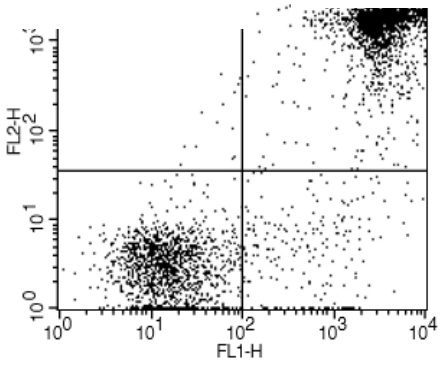
Quadrant Statistics

File: Apoptosis 12-12-14.012
Patient ID:
Panel: Apoptosis JK
Gate: No Gate
Total Events: 4020

Sample ID:
Tube: #13 BPA 1.56ug/mL 1
Acquisition Date: 12-Dec-14
Gated Events: 4020

Quad	Events	% Gated	% Total
UL	13	0.32	0.32
UR	1757	43.71	43.71
LL	2027	50.42	50.42
LR	223	5.55	5.55

2-14.(



Quadrant Statistics

File: Apoptosis 12-12-14.006
Patient ID:
Panel: Apoptosis JK
Gate: No Gate
Total Events: 4050

Sample ID:
Tube: #7 BPA 25ug/mL 1
Acquisition Date: 12-Dec-14
Gated Events: 4050

Quad	Events	% Gated	% Total
UL	15	0.37	0.37
UR	2399	59.23	59.23
LL	1432	35.36	35.36
LR	204	5.04	5.04

Figure 5. Proliferation of T-cells (A) and detection of apoptosis (B & C) after *in vitro* BPA exposure of splenocytes to the concentrations ranging from 1.56 to 100 µg/mL. (A) Cultures of splenocytes from untreated C57BL/6J mice were exposed to BPA *in vitro,* in the absence (non-stimulated) or presence (stimulated) of 3 µg/mL ConA. Alamar Blue reagent was added to the cultures after 72 hours incubation and absorbance was measured at 570nm using spectrophotometer. Data are shown as mean ±SEM, with significance determined by student's t-test (*$p \leq 0.05$). (B) Detection of apoptosis in C57BL/6J splenocytes cultured for 24 hours with BPA concentrations of 1.56, 12.5, and 25 µg/mL was performed by Annexin-V FITC assay. Apoptotic events were quantified by use of flow cytometry; alive cells were detected as Annexin-V $FITC^-$ PI^-, whereas apoptotic cells were Annexin-V $FITC^+$ PI^+. Data are shown as mean ±SEM, with significance determined by student's t-test (*$p \leq 0.05$ between the marked groups). (C) Representative dot plots of apoptotic cells obtained by flow cytometry; percentages of apoptotic and alive cells of control (left) 1.5 µg/mL BPA-exposed (right) and 25 µg/mL BPA-exposed cells (bottom). Apoptotic cells (Annexin-V $FITC^+$ PI^+) were shown in upper right quadrant, while the alive cells (Annexin-V $FITC^-$ PI^-) in lower left quadrant.

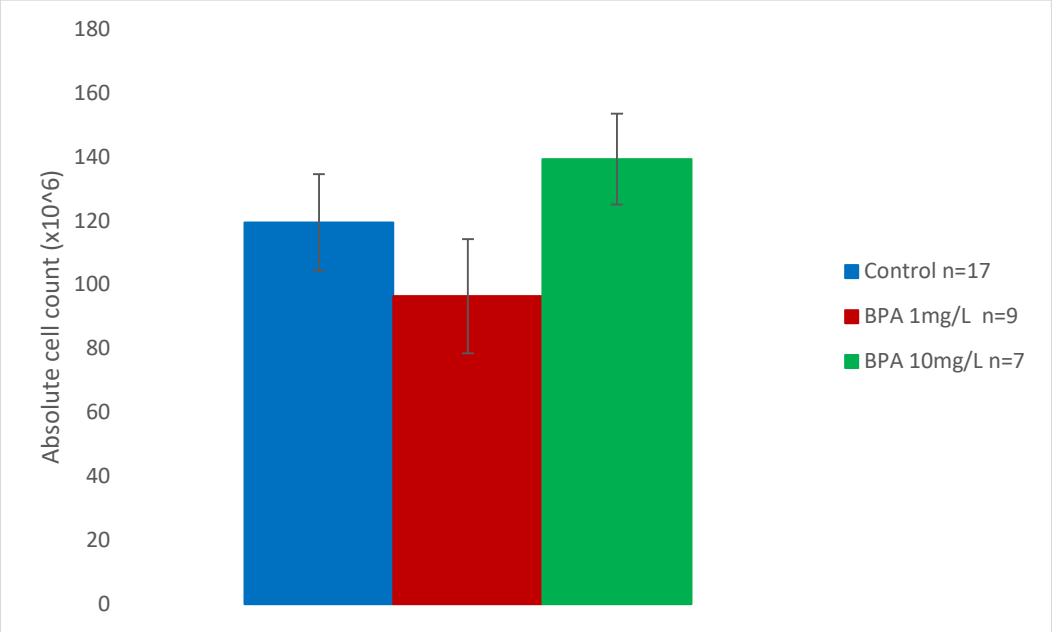
Effects of BPA Exposure on the Immune Parameters in MLDSTZ-treated C57BL/6J Male Mice During the Disease Development

Based on the ability of BPA to affect T-cell proliferation *in vitro,* next we asked would a chronic exposure to BPA affect T-cell composition and function *in vivo* during development of T1D. Four-week-old male C57BL/6J mice were exposed to low (1 mg/L)- and high (10 mg/L)-dose BPA. At nine weeks of age mice received 40mg/kg per body weight MLDSTZ injections for the induction of diabetes, and were euthanized, and splenocytes harvested at 11 or 50 days post the first STZ injection (at 10 or 16 weeks of age) to test for: total cell counts, viability of the cells, immunophenotyping of T-cell subsets and other immune cell types, proliferation of T-cells, and cytokine profiles.

The following results were obtained on day 11 post first STZ injection: The total cell counts ($x10^6$) did not significantly differ among the groups (Figure 6). Cell viability was 90.3, 91.3, and 90.5% in control, low-dose, and high-dose BPA groups respectively, which suggests that these BPA doses did not induce toxic effects *in vivo*. Immunophenotyping of immune cells,

in terms of percentages, revealed a significant decrease in entire T-cell population ($CD3^+$) ($p=4.7x10^{-3}$), including T helpers ($CD4^+$) ($p=9.2x10^{-3}$), T cytotoxic cells ($CD8^+$) ($p=4.9x10^{-2}$), natural killer cells ($p=1.1x10^{-2}$), and a significant increase in B-cells (B220) ($p=5.4x10^{-3}$) in the low-dose group compared to the control (Figure 7A). Immunophenotyping presented by total cell counts (Figure 7B) showed a significant decrease in T-helpers ($p= 1.4x10^{-2}$), and a similar trend of reduction in the cell types observed in the low-dose group when the percentages were analyzed (Figure 7A). In contrast, there were no differences observed in either percentages or the absolute cell numbers in the immune cells obtained from high-dose group compared to the controls (Figure 7A & B). Analysis of T-cell proliferation post BPA treatment *ex vivo* showed a significant increase ($p=1.1x10^{-3}$) only in the low-dose group in non-stimulated splenocytes, without a difference observed in proliferation in the high-dose group, compared to the controls (Figure 8). Investigation of the cytokines, obtained from splenocytes of the low-dose BPA-exposed mice, showed a trend of increase in inflammatory cytokines, such as TNF-α, IL-6, and IFN-γ, and a trend of decrease in IL-4, IL-2, IL-17A, compared to controls. Interestingly, significant decrease was observed in pathogenic cytokines TNF-α and IFN-γ and a trend of decrease in all other cytokines tested, in high-dose group, compared to controls and low-dose BPA ($p=4x10^{-2}$ and $p=9x10^{-3}$ compared to control and low-dose BPA group, respectively, for TNF-α; $p=4x10^{-2}$ compared to low-dose BPA group for IFN-γ) (Figure 9).

A.



B.

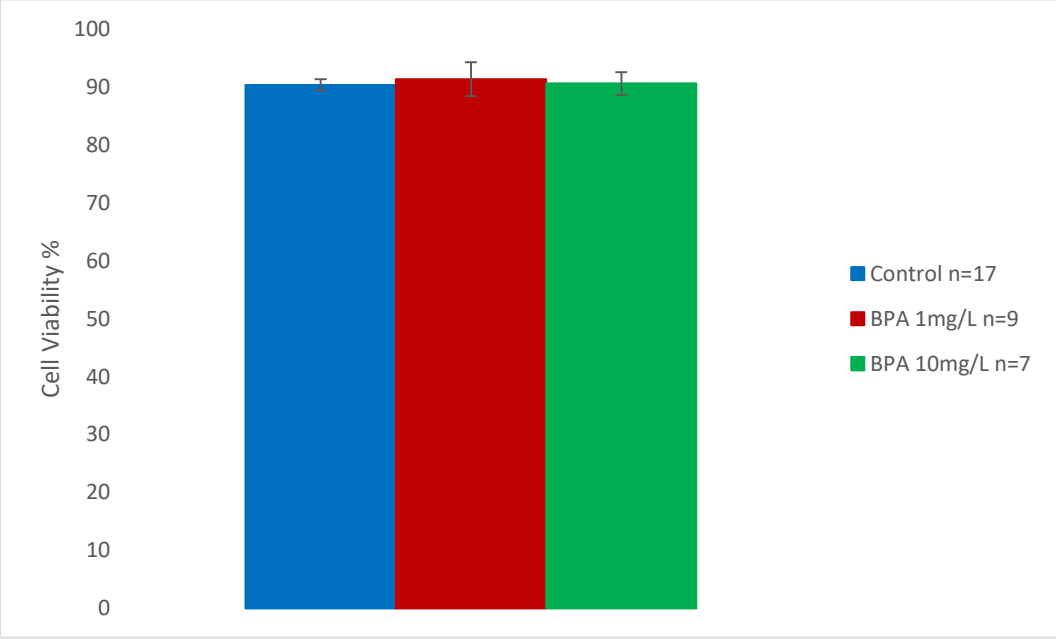
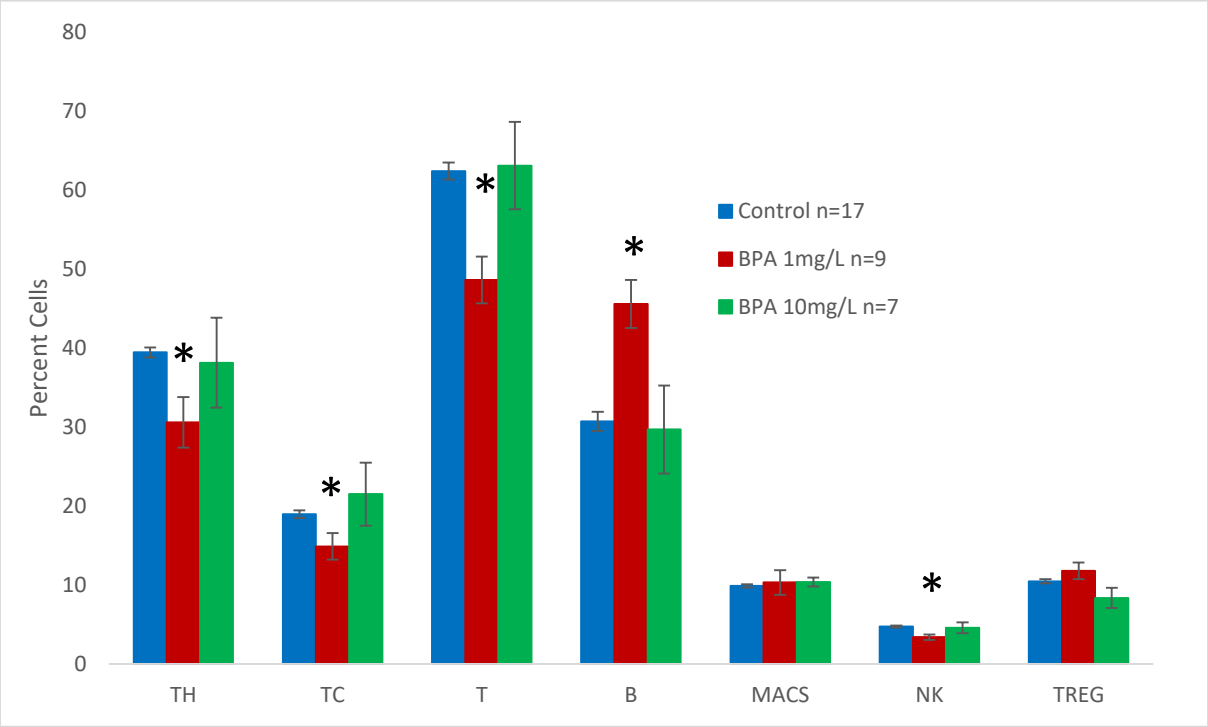


Figure 6. Total splenic cell counts (x10^6) (A) and viability (B) in BPA-exposed MLDSTZ-treated C57BL/6J male mice on day 11 post first STZ injection. Mice were treated with either 1 mg/L,

10 mg/L BPA, or control buffer dissolved in drinking water from four to 16 weeks of age. Five MLDSTZ injections were administered at nine week of age. Total cell counts and viability were obtained by the use of Trypan Blue exclusion method. Cell viability was determined by calculating the average viability percentages based on total cell counts (alive cells/total cells x100). Data are presented as mean ±SEM; t-test performed.

A.



B.

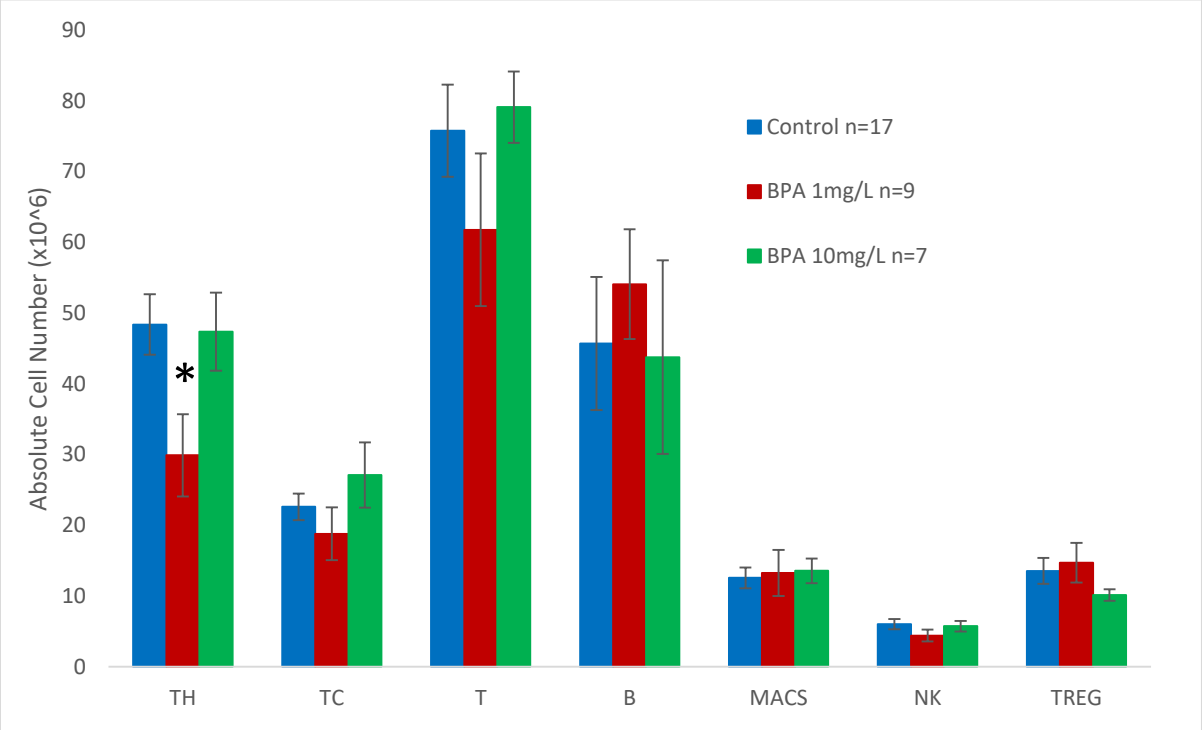


Figure 7. Immunophenotyping of splenocytes in BPA-exposed MLDSTZ-treated C57BL/6J male mice on day 11 post first STZ injection. Percentages (A) and total cell counts ($x10^6$) (B) of splenic T helpers (TH), T cytotoxic (TC), total T-cells (T), B-cells (B), macrophages (MACS), natural killer cells (NK), and T-regulatory cells (TREG) are presented above. C57BL/6J mice were treated with BPA and control water solution as described in the legend of Figure 6. Splenocytes were stained with antibodies against particular cell markers of aforementioned immune cells and analyzed by flow cytometry. Data are presented as mean ± SEM; t-test (*p ≤0.05 compared to controls for a particular cell type).

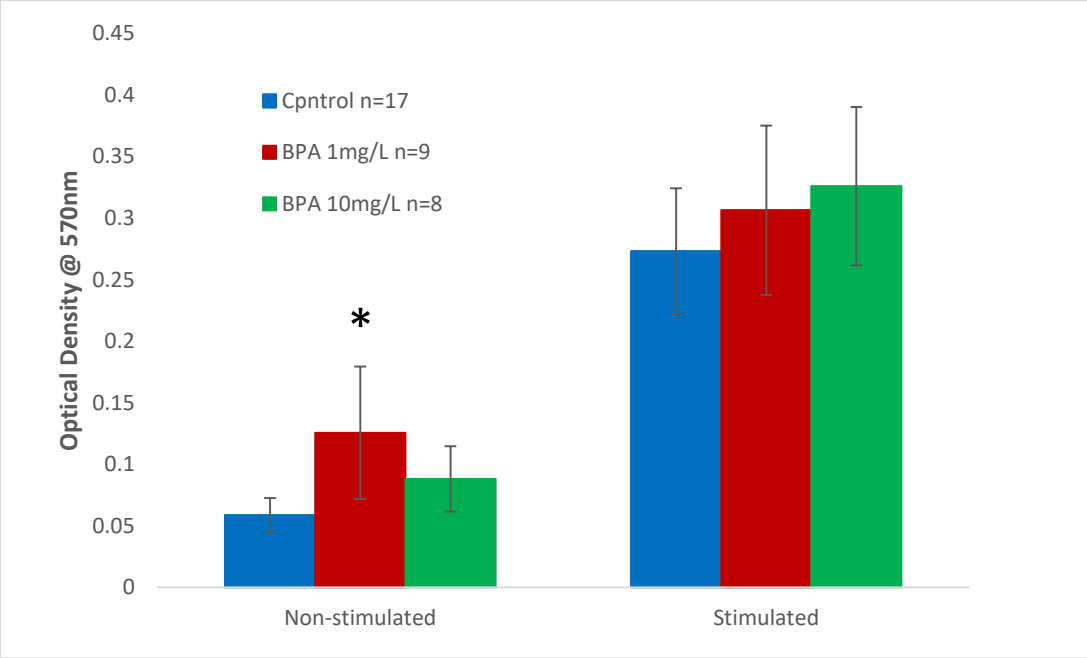


Figure 8. Proliferation of T-cells obtained from BPA-exposed MLDSTZ-treated C57BL/6J male mice on day 11 post first STZ injection after exposure to either 1 mg/L, 10 mg/L BPA, or control buffer. Mice were treated with BPA and control water solution as outlined in the legend of Figure 6. Splenocytes were cultured in the absence or presence of 3 µg/mL ConA (non-stimulated or stimulated, respectively). Alamar Blue colorimetric assay was used to measure the proliferation of T-cells and absorbance determined at 570nm. Data are presented as mean ± SEM; t-test (*$p \leq 0.05$ compared to BPA-treated groups).

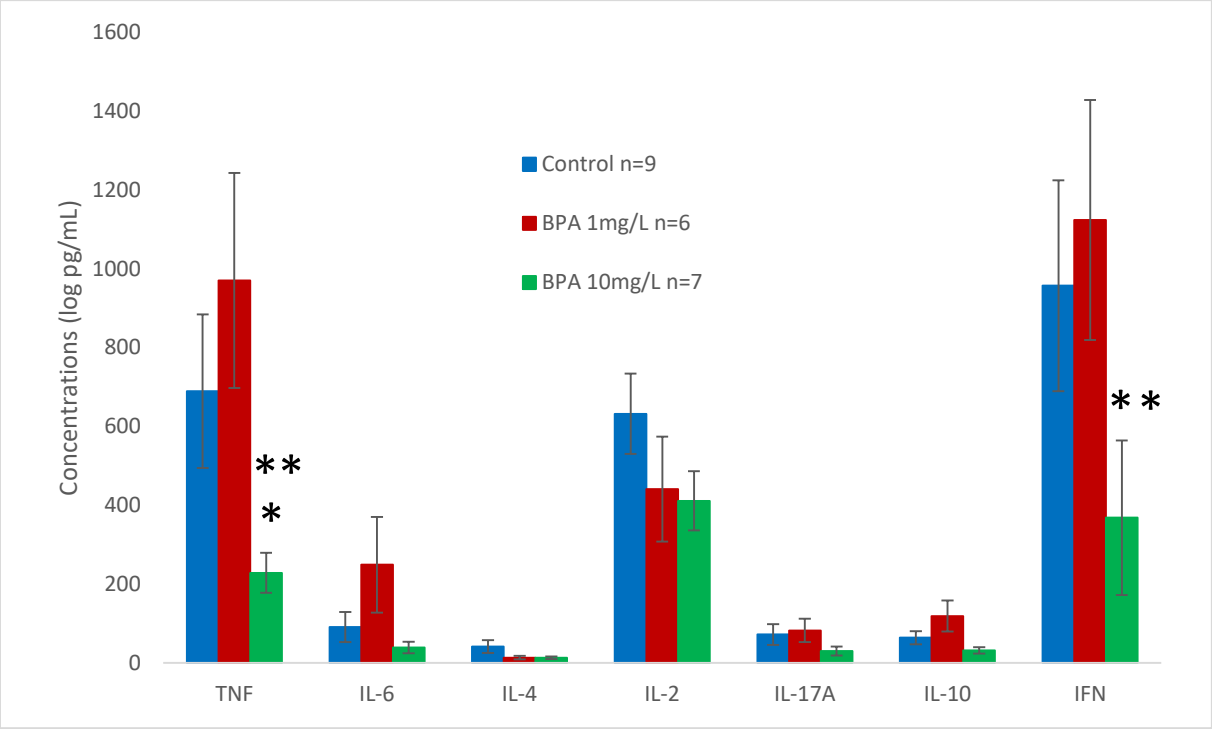


Figure 9. Quantification of cytokine levels in BPA-exposed MLDSTZ-treated C57BL/6J male mice on day 11 post first STZ injection. Mice were treated with BPA or control water solution as mentioned in the legend of Figure 6. After 48 hours of culture of splenocytes in the presence of 3 µg/mL ConA, supernatants were collected and analyzed using a CBA mouse $T_{H1,}$ $T_{H2,}$ T_{H17} kit that allows quantification of TNF-α, IL-6, IL-4, IL-2, IL-17A, IL-10, and IFN-γ**.** Data are presented as mean ± SEM; t-test (*, **p≤ 0.05 compared to control and BPA 1 mg/L groups, respectively).

The following results were obtained on day 50 post first STZ injection: Mice were euthanized at the experimental endpoint and splenocytes were harvested to test for total cell counts, viability of the cells, immunophenotyping of T-cell subsets and other immune cell types, proliferation, and cytokine profiles. Total number of splenocytes ($x10^6$) were not significantly different in both low- and high-dose BPA-exposed mice compared to controls (Figure 10A). Cell viability was 85.5, 81.3, and 91.3% in control, low-dose, and high-dose BPA groups, respectively (Figure 10B). Whereas a trend of a reduction in the percentages of CD3+, Th and Treg cells was observed in 1 mg/L BPA-exposed mice, a statistical significance was not reached

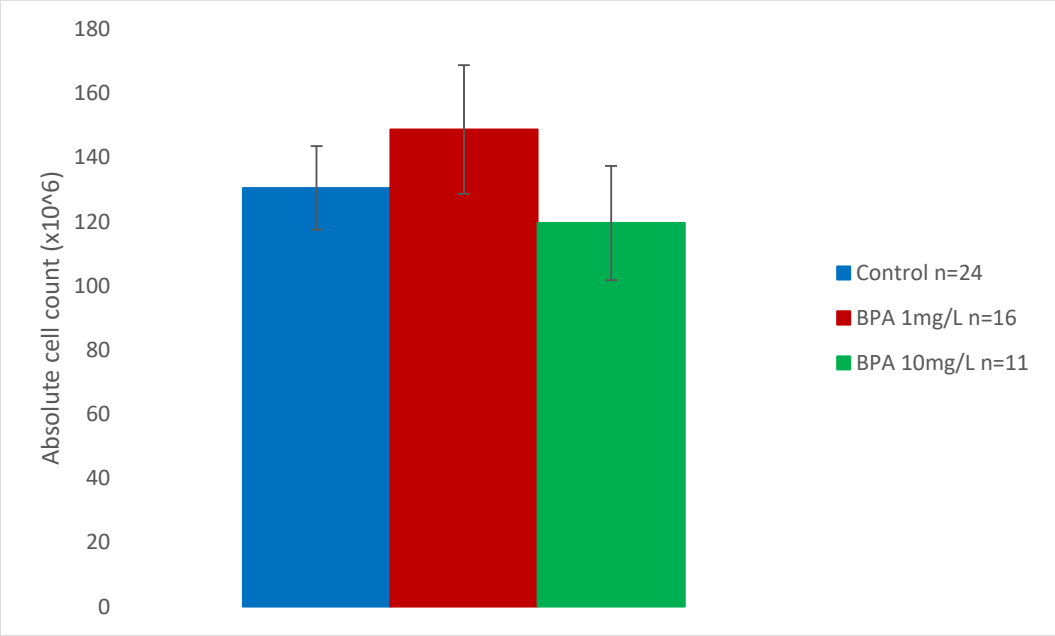
neither in the terms of percentages (Figure 11A) nor absolute cell numbers in any of the studied cell types compared to controls (Figure 11B). There were no significant differences observed in the immunophenotypes of splenocytes in terms of the percentages (Figure 11A) or the absolute numbers (Figure 11B) in high-dose BPA-exposed mice compared to the controls. Analysis of T-cell proliferation, interestingly, showed significant increase in a high-dose group compared to the control ($p= 1.2x10^{-3}$), as well as to the low-dose BPA-treated group ($3.4x10^{-3}$) in non-stimulated cultures. In stimulated cultures, while a trend of increased proliferation was observed in the high-dose group, a significant suppression of proliferation was found in the low-dose group compared to control ($p=1.7x10^{-3}$) and to high-dose BPA-exposed group ($p=1.5x10^{-5}$) (Figure 12).

Investigation of the cytokines, obtained from the splenocytes of the BPA-exposed mice, showed a trend of increase in cytokines TNF-α, IL-6 and IL-17A in both low- and high-dose groups, and increase in IFN-γ in the high-dose group. Interestingly IL-2 was reduced in both low- and high-dose groups compared to controls. However, no significant differences were found between any of the treatment groups and controls on day 50 post first STZ injection (Figure 13).

Considering the immunological parameters, results obtained from day 11 post the first STZ injection in low-dose BPA treated group showed a significant reduction in the percentages of all T-cells, including T helpers and cytotoxic T-cells, with a most prominent reduction reflected in the absolute numbers of helper T-cells. Function-wise, spontaneous, not ConA-induced, proliferation of splenocytes, indicated other cell types being affected by low-dose BPA exposure besides T-cells, probably B-cells, as their percentage was found to be significantly increased. Interestingly, cytokine analysis revealed that these lower amounts of T-cells exhibited a tendency towards elevated secretion of proinflammaory cytokines. In contrast, high-dose BPA

exposure affected neither T-cell population percentages/numbers nor proliferative capacity. However, a reduction in the levels of all the studied cytokines, with a significance reached in IFN-γ and TNF-α levels, was observed in the high-dose BPA group. At the end of experimental period, on day 50 post first STZ injection, when mice exhibited a full-blown diabetes, there were no significant differences between the either low- or high-dose-exposed BPA groups and control in the percentages/absolute numbers of the T-cells, or any other immune cell types studied. Interestingly, while high-dose BPA-treated mice showed increased spontaneous proliferation of splenocytes, ConA-simulated proliferation was significantly suppressed in the low-dose treatment group. Cytokine analysis did not show a significant difference in any of the studied cytokines in both BPA treatment groups. However, it is worth noticing that the splenocytes obtained from high-dose BPA-exposed mice increased their cytokine secretion to the control levels, not exhibiting decreased amounts of proinflammatory cytokines, observed in the beginning of the disease development (on day 11 post first STZ injection).

A.


180
160
140
120
100
80
60
40
20
0
Absolute cell count (x10^6)
Control n=24
BPA 1mg/L n=16
BPA 10mg/L n=11

B.

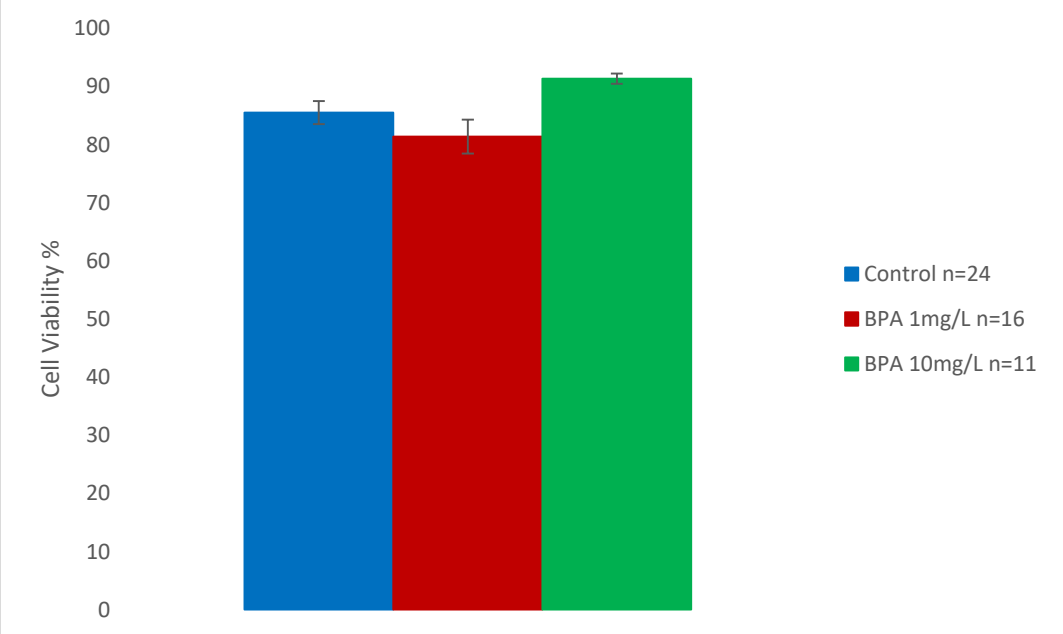
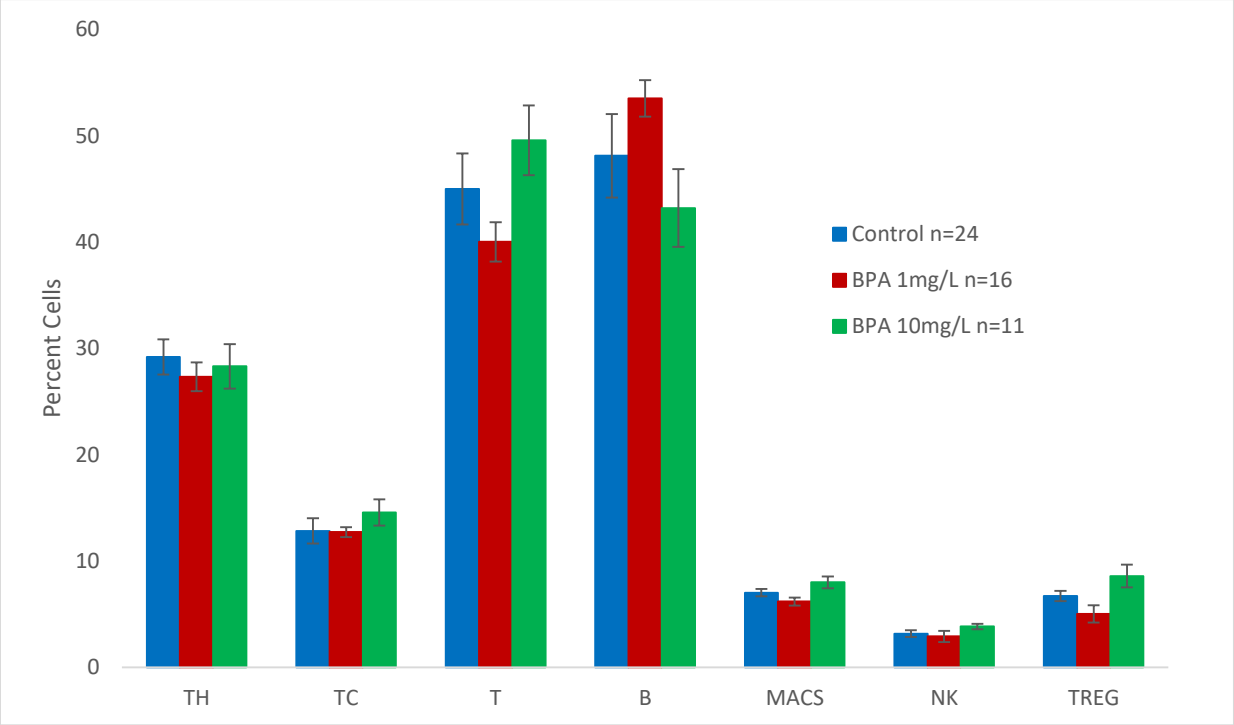

100
90
80
70
60
50
40
30
20
10
0
Cell Viability %
Control n=24
BPA 1mg/L n=16
BPA 10mg/L n=11

Figure 10. Total splenic cell counts ($x10^6$) (A) and viability (B) of BPA-exposed MLDSTZ-treated C57BL/6J male mice on day 50 post first STZ injection. Mice were treated with either control buffer, 1 mg/L, or 10 mg/L BPA dissolved in drinking water, as mentioned in the legend of Figure 6. Total cell counts and viability were obtained by the use of Trypan Blue exclusion method. Cell viability was determined by calculating the average viability percentages based on total cell counts (alive cells/total cells x100). Data are presented as mean ±SEM; t-test was performed.

A.



B.

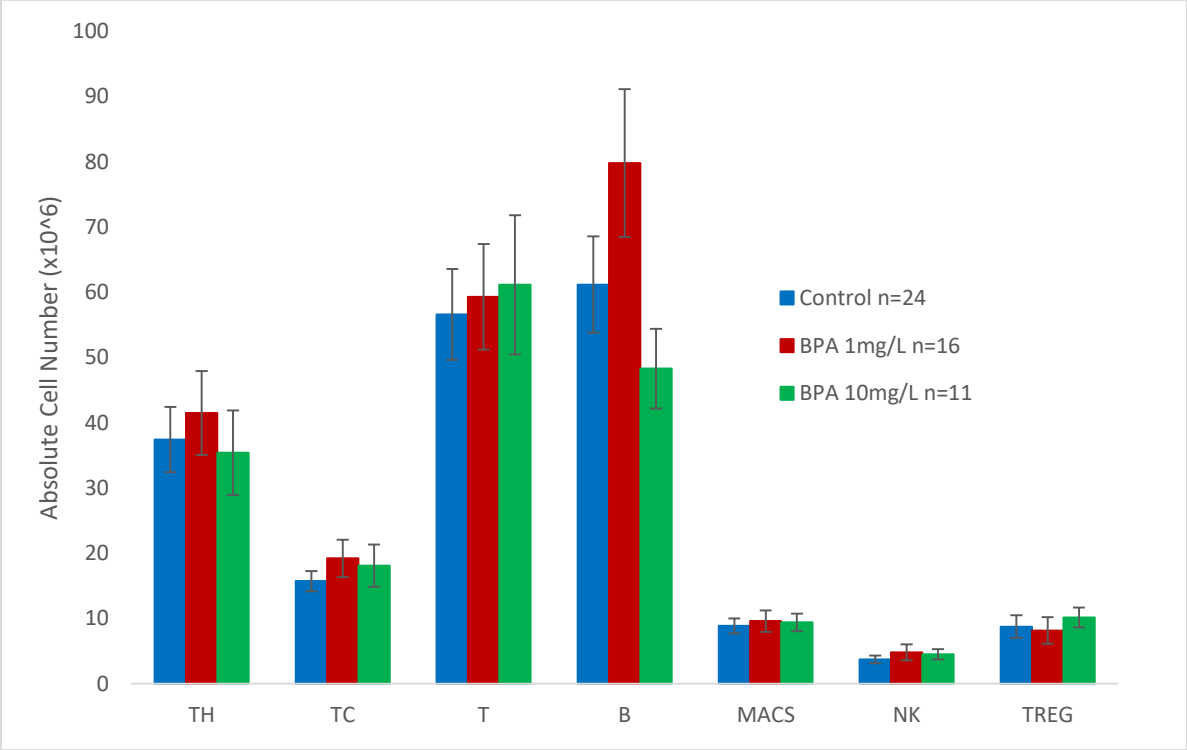


Figure 11. Immunophenotyping of splenocytes in BPA-treated C57BL/6J male mice on day 50 post first STZ injection. Percentages (A) and total cell counts ($x10^6$) (B) of splenic T helpers (TH), T cytotoxic (TC), total T-cells (T), B-cells (B), macrophages (MACS), natural killer cells (NK), and T-regulatory cells (TREG) are presented above. C57BL/6J mice were treated with BPA and control water solution as described in the legend of Figure 10. Splenocytes were stained with antibodies against particular cell markers of aforementioned immune cells and analyzed by flow cytometry. Data are presented as mean ± SEM; t-test was performed.

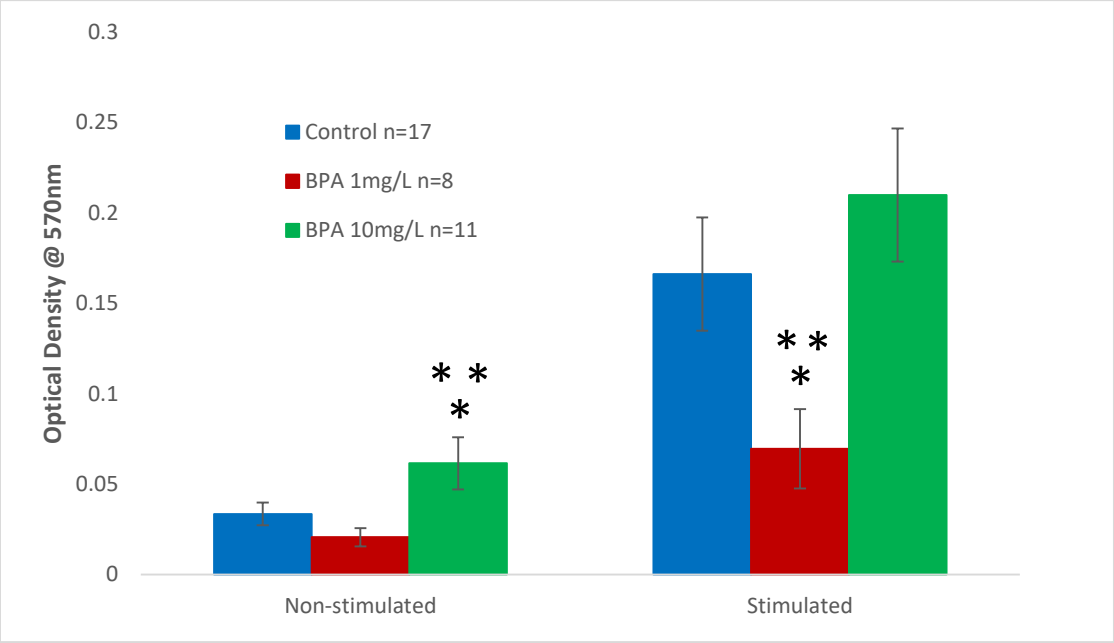


Figure 12. Proliferation of T-cells obtained from BPA-treated C57BL/6J male mice on day 50 post first STZ injection after exposure to either control buffer, 1 mg/L, or 10 mg/L BPA. Mice were treated with BPA and control water solution as outlined in the legend of Figure 10. Splenocytes were cultured in the absence or presence of 3 µg/mL ConA (non-stimulated or stimulated respectively). Alamar Blue colorimetric assay was used to measure the proliferation of T-cells and absorbance determined at 570nm. Data are presented as mean ± SEM; t-test (*, **$p \leq 0.05$ compared to control and BPA-treated groups, respectively).

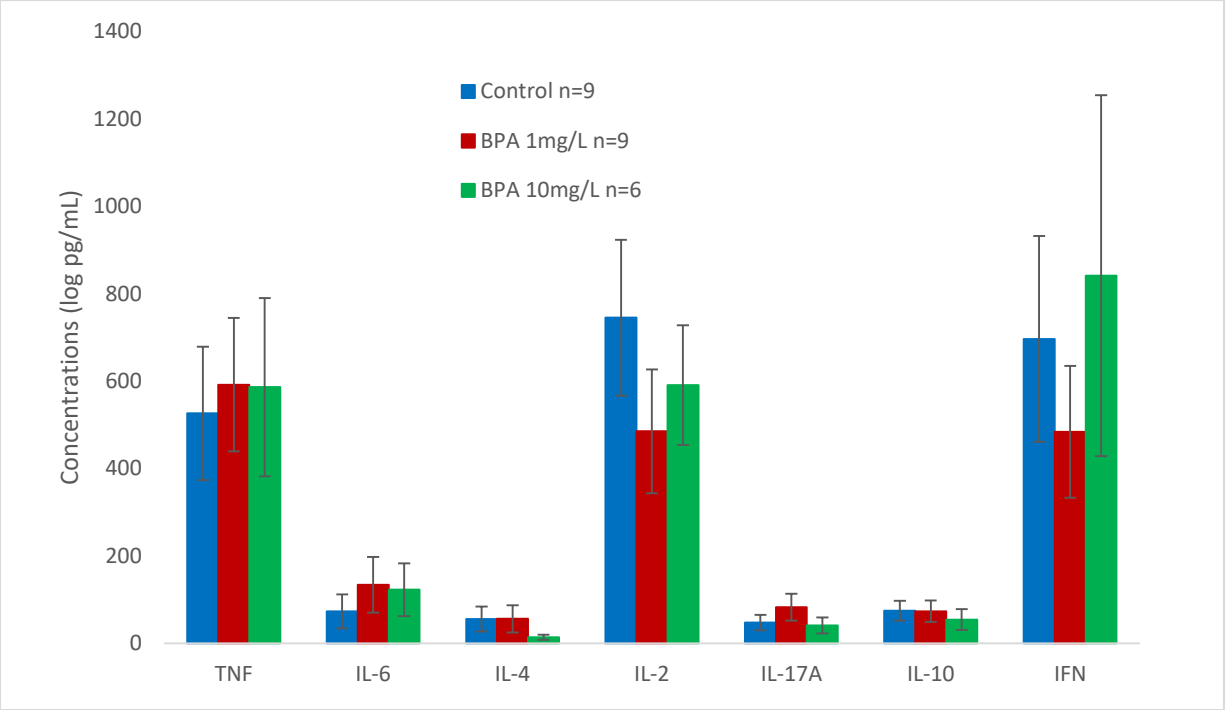


Figure 13. Quantification of cytokine levels in BPA-exposed MLDSTZ-treated C57BL/6J male mice on day 50 post first STZ injection. Mice were treated with BPA or control water solution as mentioned in the legend of Figure 10. After 48 hours of culture of splenocytes in the presence of 3 µg/mL ConA, supernatants were collected and analyzed using a CBA mouse T_{H1}, T_{H2}, T_{H17} kit that allows quantification of TNF-α, IL-6, IL-4, IL-2, IL-17A, IL-10, and IFN-γ. Data are presented as mean ± SEM; t-test was performed.

Chapter V- DISCUSSION

To the best of our knowledge, this study has investigated for the first time the effect of BPA on the development of T1D in MLDSTZ-induced C57BL6/J mouse model in the context of T-cells. We showed that long-term exposure of MLDSTZ-treated C57BL/6J mice to low, as well as high BPA dose, potentiated diabetes development and insulitis levels. Whereas both doses of BPA exhibited immunomodulatory activities during development of chemically-induced autoimmune T1D, their mechanism of diabetogenic action seems to be divergent. BPA exposure exhibited a prominent alteration in T-cell population levels, especially helper T cells, and a trend of increased production of proinflammatory cytokines early on during the diabetes development. These results suggest that low-dose BPA treatment initiates diabetogenesis through the alteration of T-cell function and potentiation of a Th1-like immune response. However, a mechanism of the early diabetogenic action of a high-dose BPA treatment, which did not affect T-cell populations, along with even suppressed production of proinflammatory cytokines, did not seem to be T-cell-associated, assuming known pathogenicity of proinflammatory responses in early stages of diabetogenesis. Thus, these data suggest that high-dose BPA potentiated diabetes development via action on other cell types, probably pancreatic beta cells. Interestingly, even at the end of experimental period, when both groups of BPA-exposed mice exhibited already developed diabetes and lack of prominent disturbances of immune system, low- and high-dose BPA treatments still showed different effects on the immune system; low-dose BPA suppressed proliferation of T-cells, while high-dose BPA treatment induced an increase in secretion of proinflammatory cytokines, not in the terms of comparison to control values, but compared to the suppressed levels observed in this treatment group in the beginning of disease development.

Overall, these data confirm diabetogenic potential of BPA and show its divergent dose-associated mechanism of action; low-dose BPA seems to potentiate diabetes development through the alteration of T-cell populations and their function during the early stage of diabetes development, while high-dose BPA's diabetogenic potential does not rely initially on T-cell disturbances, but later on probably potentiates disease severity through its immunomodulatory effects.

Although in the recent years a few experimental studies have investigated the question whether BPA affects the development of T1D in animal models, none of these studies, however, answered the mechanism behind the induction of T1D post exposure to BPA in terms of immune cells known to mediate the disease development, T-cells. A study by Bodin et al, showed increased insulitis and a tendency towards higher diabetes incidence (although confirmation of statistical significance was lacking) in adult NOD mice long-termly exposed to 1mg/L BPA (Bodin, Bolling,Samuelsen, et al., 2013), as well as in NOD mice transmaternally exposed to 10mg/L BPA (Bodin, Blling, Becher, et al., 2013). Though T-cell proliferation were investigated in the later study (Bodin, Bølling, Samuelsen, et al., 2013), no differences found between groups, showing no compelling evidence that a BPA action in potentiation of T1D was T-cell mediated. The mechanism of BPA action in both Bodin's studies, performed in adult, as well as transmaternally-exposed NOD mice, was attributed to induction of apoptosis of pancreatic beta cells and residential macrophages (Bodin, Bølling, Samuelsen, et al., 2013; Bodin, Bølling, Becher, et al., 2013). Surprisingly, whereas a trend of T1D acceleration was observed in mice exposed to 1 mg/L BPA, high-dose 100 mg/L BPA exposure, quite opposite, prevented diabetes development, suggesting a divergent action of low vs. high BPA dose (Bodin, Bølling,

Samuelsen, et al., 2013). Our results supported Bodin's low-dose (1 mg/L) BPA effects on T1D development. Whereas we did not study 100 mg/L dose, exposure to 10 mg/L BPA showed a potentiation of T1D as well. Interestingly, whereas we observed a divergent mechanism of diabetogenic action in 1 mg/L- vs. 10 mg/L-treated mice, Bodin et al. found same mechanism of action, for low (1 mg/L) and high (100 mg/L) BPA doses, in terms of pancreatic beta cells and residential macrophages apoptosis, regardless their opposite effects observed on T1D development (Bodin, Bølling, Samuelsen, et al., 2013).

Since type 1 diabetes is primarily characterized by hyperglycemia, the first part of our study investigated, besides T1D incidence, the glycemia levels in MLDSTZ-treated C57BL6/J mice during chronic exposure to low and high-doses BPA for 12 weeks. Overall glycemia levels showed a trend of increase throughout the study in the high-dose BPA exposed group, compared to low-dose and control groups. Significant increase in diabetes incidence, observed in both high-dose and low-dose BPA-exposed groups, was further supported by higher severity of insulitis level.

Among multitude of animal models, chemically-induced C56BL/6J mouse model of T1D was used for all the components of this study. This model provided an excellent opportunity to investigate the effects of potentially toxic substances (such as BPA) in an environment where insulin-producing beta cells have been already partially damaged by STZ. This mimics a potential scenario present in humans who have already been exposed to other beta cell damaging substances (drugs, food, other chemicals) or pathogens (rubella virus, mumps virus, etc.) before or along the exposure to environmental pollutants.

One of the very popular epidemiological studies, conducted by Center for Disease

Control (CDC), showed that detectable level of BPA traces was found in urine samples in nearly 93% of the human participants of the National Health & Nutritional Examination Survey (EPA, 2010). This indicates greater exposure levels of humans to BPA via not only food and water, but also through dust, air, and dermal contact. Doses of BPA, 1 and 10 mg/L/day, chosen for our exposure studies, were determined primarily based on the studies published by Bodin et al (Bodin, Bølling, Samuelsen, et al., 2013; Bodin, Bølling, Becher, et al., 2013). Table 2. shows that the concentrations of BPA we used fall in the range of BPA found in the environment in water bodies. In addition, Table 3. shows that our daily doses are comparable with BPA doses used in other studies and in different animal experimental models. Thus, both the doses of BPA 1 mg/L (160 µg/kg) and 10 mg/L (1600 µg/kg) used for this study fall within the range of other animal studies and environmental exposures. Interestingly, European Food Safety Authority (EFSA) has recently (EFSA, 2015) lowered the estimated safe level, known as the tolerable daily intake (TDI), from 50 µg/kg (EPA, 2011) to 4µg/kg per day. Even this TDI is actually "temporary" TDI, until the results of ongoing toxicology research can be incorporated in the evaluation (EFSA, 2015). Based on this new TDI, one can conclude that doses of BPA used in our studies are too high to be considered as relevant for human exposure. However, EFSA's report emphasized uncertainties surrounding potential health effects of BPA on different body systems, including immune system (EFSA, 2015).

Since T1D is a T-cell-mediated and -dependent disease, *in vitro* effects of BPA on T-cell function were investigated as well. When splenocytes from untreated mice were stimulated by T-cell mitogen ConA and simultaneously exposed to a serial dilution of BPA~~,~~ higher concentrations of BPA, from 6.25 to 100µg/mL, suppressed T-cell proliferation. Apoptosis

analysis revealed that high BPA concentrations significantly increased apoptotic cells, suggesting that BPA has the ability to reduce T-cell proliferation *in vitro* by inducing apoptosis. This further supports that BPA could directly impair the function of T-cells. In line with our results, other studies showed that BPA induced apoptotic cell death in different cell types. For example, in 2013 Lin et al found that exposure to BPA in concentrations 0.2 and 2.0µM, (similar to 25µg/mL (0.1µM) in our study) affected insulin secretion and induced apoptosis in rat insulinoma cells (Lin et al., 2013). In addition to this, Bodin et al. showed apoptotic cell death of beta cells and residential macrophages in NOD mice exposed to BPA either in adult stage of life or transmaternally (Bodin, Bølling, Samuelsen, et al., 2013; Bodin, Bølling, Becher, et a., 2013).

Given the ability of BPA to affect T-cell proliferation in vitro, this study also investigated the immune system-related effects of BPA during the exposure of MLDSTZ-treated C57BL6/J male mice to BPA. Total cell counts, viability of cells, immunophenotyping of T-cell subsets and other immune cell types (B-cells, macrophages, NK cells), proliferation of T-cells, and cytokine profiles were analyzed at two different time points; 11 days and 50 days post the first STZ injection. Early on during T1D development in MLDSTZ model, on day 11 post the first STZ injection, findings showed reduced proportions of T-cells and their subpopulations, such as helpers and cytotoxic T-cells, and even reduction in the absolute number of T-cells, especially helper T-cells, in the low-dose BPA-exposed mice. In addition to this, a trend of increase in pathogenic cytokines was observed in a low-dose BPA-exposed group compared to controls, indicating a potential increase in Th-type immune response. Since the alterations in the composition and function of T-cells were observed, these findings supported our initial hypothesis that low-dose BPA exposure accelerates the disease development through T-cell-

mediated mechanism during the early stages of disease development. Interestingly, these results were not observed in the high-dose BPA-exposed group, which showed neither a decrease in T-cell populations, nor a trend of increase in proinflammatory cytokines. Quite opposite, a significant reduction of IFN-γ, and TNF-α was observed in those mice. On day 50 post the first STZ injection, same immunological parameters were evaluated as earlier. There were no differences observed in the composition of T-cells in both groups of BPA-exposed mice. However, a significant suppression of proliferation in a low-dose group, compared to both control and high-dose group, was found. The high-dose BPA-exposed mice exhibited a trend of increased T-cell proliferation and an interesting finding of considerable increase in pathogenic cytokines, compared to suppressed levels observed in early stages of disease development.

It has been shown that hyperglycemia per se can suppress T-cell function; T-cells cultured in high glucose concentrations in the absence of insulin displayed decreased proliferation as a result of activation of protein kinase A (Sakowicz-Berkiewicz, Kocbuch, Grden, Szutowicz, and Pawelczyk, 2006). Considering earlier diabetes onset and higher glycemic values observed in high-dose BPA-treated mice, a hyperglycemic influence might be implied as a cause of "weak" immune responses, reflected in diminished secretion of cytokines, in comparison to low-dose treatment group. However, taking into account differential immune profiles and immune responses observed in low- vs. high-dose-exposed mice and glycemic effects, one would expect potentiation of those findings with prolonged duration of disease and increased hyperglycemia values towards the end of experiment. In contrast, on day 50 post first STZ injection, when average hyperglycemia was 320mg/dL and more than 400mg/dL in low- and high-dose BPA-exposed groups, respectively, high-dose treatment group exhibited more

robust production of pathogenic cytokines compared to suppressed levels observed at the earlier time point.

In this study, to scrutinize the effects of low-dose BPA on the development of T1D in MLDSTZ-induced C57BL/6J mice, the overall cytokine analysis showed a tendency of increase in proinflammatory cytokine production, specifically leaning towards Th1-type immune response. Previously published results support our findings regarding potentiation of Th1-type response by BPA treatment. Thus, prenatal BPA exposure of mice led to upregulation of Th1 responses in adulthood (Shin et al., 2004 & Yoshino et al., 2004). In another study performed to test the impact of BPA on the immune system, using T-cell receptor transgenic mice, the cytokine profile analysis showed that administration of BPA in the range of 1.5-1.8 mg/kg bodyweight augmented Th1 immune response with increase in IFN-γ (Goto, Takano- Ishikawa, & Li, 2007 and Frederick, Vom, & Hughes, 2005).

Furthermore, a significant up-regulation of Th1 immune response with an increase of IFN-γ was observed by Shin et al, 2004, using 3000 µg/kg dose of BPA. Alizadeh et al. found augmented Th1 immune response in female BALB/c mice exposed to 100 nM BPA through drinking water (Alizadeh et al., 2006), further supporting our findings. In addition, a study by Huimin et al. showed that prenatal exposure to BPA not only decreased the percentage of Tregs, disrupting the immunological balance, but also potentiated development of both Th1 and Th2 immune responses (Huimin et al., 2008).

The mechanism through which BPA affects T-cells may be explained via the estrogenic effects of BPA. BPA is known to be an estrogen-mimicking endocrine disruptor that shows estrogenic activity, which plays a role in activating hyperactive immune responses that could

occur in autoimmune pathophysiology. This is important because increased circulating estrogens have demonstrated close association with greater autoimmune activity (Chailurkit, Aekplakorn, & Ongphiphadhanakul, 2013), and estradiol in vitro has shown to upregulate Th1, but not Th2 responses (Sahin et al., 2001 & 2016). In addition, Canesi et al, found that BPA's binding to estrogen receptors leads to dysfunction of pathways (Canesi, Betti, Lorusso, Ciacci, & Gallo, 2005), such as mitogen activated protein kinases (MAPKs) and signal transducer and activator transcription (STAT) signaling pathways, shown to play a pivotal role in prevention of upregulation of autoreactive T-cells (Wildner & Kaufmann, 2013).

Chapter VI- CONCLUSION

In conclusion, our study showed for the first time the effects of BPA on the development of T1D in MLDSTZ model in C57BL/6 mice in terms of T-cells. The findings corroborated diabetogenic potential of BPA and showed T-cell-dependent mechanism of BPA action. Besides confirming a correlation between BPA and T1D for the public, these results provide additional information to environmental agencies. Thus, an association between BPA and TID could offer a persuasive challenge for future epidemiologic and toxicological research, as well as for regulatory institutions to limit/reduce BPA usage.

Table 1. Concentrations of BPA that are present in the environment including water bodies (mg/L) and sediments (µg/kg).

Water (mg/L)	**Sediment (µg/kg)**	**Reference**
0.5- 410	10- 190	(Fromme et al., 2002)
4- 92	10- 380	(Stachel et al., 2003)
9- 776	66- 343	(Heemken, Reincke, Stachel, and Theobald, 2001)
No data	<5- 1630	(Stachel et al., 2003)
No date	204	(Koh, Khim, Villeneuve, Kannan, and Giesy, 2002)

Table 2. Daily doses of several studies performed in various animal models and their routes of exposure in µg/kg. These are secondary references cited in Richter et al, 2007.

Animal model	**Route of exposure**	**BPA dose (µg/kg)**	**Reference**
Sprague- Dawley	Oral	40	(Ceccarelli et al., 2007)
Wistar rat	Osmotic mini-pump	25, 250	(Ramos et al., 2003)
Rat	Implant	300	(Steinmetz et al., 1997)
Wistar rat	Oral, water	1500	(Kubo et al., 2003)
Sprague- Dawley	Oral, Oil	40, 400	(Dessi-Fulgheri et al., 2002)
C57BL/6J mice	Oral, Gavage	2200	(Ryan et al., 2006)
Wistar rat	Oral	1500	(Markey et al., 2001)
CD-1 mice	Osmotic mini-pump	25, 250	(Hunt et al., 2003)
C57BL/6J mice	Oral, Oil	20, 40, 100	(Susiarjo et al., 2007)
C57BL/6J mice	Oral, Injection	20	(Ramos et al., 2001)

References

American Diabetes Association. (2014). Retrieved from National Diabetes Statistics Report: http://www.diabetes.org/diabetes-basics/statistics/

Alizadeh, M., Ota, F., Hosoi, K., Skai, T., & Satter, M. A. (2006). Altered alergic cytokine and antibody response in mice treated with Bisphenol A. *Med Invest* 53, 70-80.

Breast cancer fund. (2008, November 13). Atale of two estrogens: BPA and DES. Retrieved from http://www.breastcancerfund.org/site/pp.asp?c=kwKXLdPaE&b=3933063.

Alonso-Magdalena, P., Morimoto, S., Ripoll, C., Fuentes, E., & Nadal, A. (2006). The Estrogenic Effect of Bisphenol A Disrupts Pancreatic β-Cell Function In Vivo and Induces Insulin Resistance. *Environmental Health Perspectives*, (1). 106.

Bodin, J., Bølling, A. K., Samuelsen, M., Becher, R., Løvik, M., & Nygaard, U. C. (2013). Long-term Bisphenol A exposure accelerates insulitis development in diabetes-prone NOD mice. *Immunopharmacology & Immunotoxicology*, *35*(3), 349-358. doi:10.3109/08923973.2013.772195

Bodin, J., Bølling, A. K., Becher, R., Kuper, F., Løvik, M., & Nygaard, U. C. (2013). Transmaternal Bisphenol A Exposure Accelerates Diabetes Type 1 Development in NOD Mice. *Toxicological Sciences, 137*(2), 311-323.

Canesi, L., Betti, M., Lorusso, L. C., Ciacci, C., & Gallo, G. (2005). 'In vivo' effects of Bisphenol A in Mytilus hemocytes: modulation of kinase-mediated signalling pathways. Aquatic Toxicology, 7173-84. doi:10.1016/j.aquatox.2004.10.011

Carwile, J. L., & Michels, K. B. (2011). Urinary bisphenol A and obesity: NHANES 2003–2006. *Environmental Research, 111*825-830. doi:10.1016/j.envres.2011.05.014

Chailurkit, L., Aekplakorn, W., & Ongphiphadhanakul, B. (2014). The relationship between circulating estradiol and thyroid autoimmunity in males. European Journal Of Endocrinology, 170(1), 63-67. doi:10.1530/EJE-13-0455

De Maria, R., Todaro, M., Stassi, G., Giordano, M., Galluzzo, A., Giordano, C., & Di Blasi, F. (1994). Defective T cell receptor/CD3 complex signaling in human type I diabetes. *European Journal Of Immunology*, *24*(4), 999-1002.

Delovitch, T. L. & Singh, B. (1997). The non- obese diabetic mouse as a model of autoimmune diabetes: Immune dysregulation gets the NOD. *Immunity* 7, 727-738.

Dieckmann, D., Plottner, H., Dotterweich, S., & Schuler, G. (2005). ActivatedCD4+CD25+ T-cells suppress antigen specific CD4+ and CD8+ T-cells, but induce a suppressive phenotype only on CD4+ T-cells. *Immunology* 115:305-314.

EFSA-European Food Safety Authority. (2006). Opinion of the scientific panel on food additives, flavorings, processing aids and materials in contact with food on a request from the commission related to 2,2-bis(4-hydroxy-phenyl) propane (bisphenol A). *EFSA J* 428, 1-75.

EFSA-European Food Safety Authority. (2015). No health risk for consumers from Bisphenol A exposure. Retrieved from http://www.bfr.bund.de/cm/349/no-health-risk-for-consumers-from-bisphenol-a-exposure-the-bfr-endorses-the-conclusion-of-the-new-efsa-assessment.pdf.

Environmental working group. (2008). Timeline: BPA from intervention to phase- out. Retrieved from http://www.ewg.org/node/27023.

Environmental Protection Agency. (2010). 2010 Progress Report: An Epidemiologic Study of Time Trends and Health Effects of Persistent Organic Pollutants, Mercury and Micronutrients. Retrieved from https://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/display.abstractDetail/abstract/9137/report/2010

Environmental Protection Agency. (2011). 2011 Action Plan Fact Sheet: A Summary of All Chemicals Action Plans Issued by EPA. Retrieved from https://www.epa.gov/sites/production/files/2014-11/documents/overview.pdf

Environmental Protection Agency. (2015). 2015 Excutive Summary: Bisphenol A Alternatives in Thermal Paper. Retrieved from https://www.epa.gov/sites/production/files/2015-09/documents/bpa_es.pdf

Frederick S. vom, S., & Hughes, C. (2005). An Extensive New Literature concerning Low-Dose Effects of Bisphenol A Shows the Need for a New Risk Assessment. *Environmental Health Perspectives*, (8). 926.

Fromme, H., Küchler, T., Otto, T., Pilz, K., Müller, J., & Wenzel, A. (2002). Occurrence of phthalates and bisphenol A and F in the environment. *Water Research*, 361429-1438. doi:10.1016/S0043-1354(01)00367-0

Graham, K. L., Sutherland, R. M., Mannering, S. I., Zhao, Y., Chee, J., Krishnamurthy, B., & ... Kay, T. H. (2012). Pathogenic mechanisms in type 1 diabetes: the islet is both target and driver of disease. *The Review Of Diabetic Studies: RDS*, *9*(4), 148-168. doi:10.1900/RDS.2012.9.148

Global Industry Analysts. (2013). Bisphenol A-a global strategic business report 1-178, SKU: GJOB5105679.

Goto, M., Takano- Ishikawa, Y., & Li, X. (2007). Orally administered Bisphenol A disturbed antigen specific immunoresponses in the naïve condition. *Bio science, Biotechnology and Biochemistry*, vol 71, no 9, pp. 2136-2143.

Guiñazú, N., Pellegrini, A., & Giordanengo, L. (2004). Immune response to a major Trypanosoma cruzi antigen, cruzipain, is differentially modulated in C57BL/6BL/6 and BALB/c mice. *Microbes Infect* 2004; 6:1250–1258.

Heemken, O.P., Reincke, H., Stachel, B. & Theobald, N. (2001) 'The occurrence of xenoestrogens in the Elbe river and the North sea', *Chemosphere*, Vol. 45, pp.245–259

Herold, K., Vezys, V., Buckingham, F., & Baumann, E. (1997). Expression and immune response to islet antigens following treatment with low doses of streptozotocin in H-2(d) mice. *Journal of Autoimmunity*, 10(1), 17-25. doi:10.1006/jaut.1996.0108

Howdeshell, K. L., Hotchkiss, A. K., Thayer, K. A., Vandenbergh, J. G., & vom Saal, F. S. (1999). Exposure to bisphenol A advances puberty. *Nature, 401*(6755), 763-764.

Huimin, Y., Takamoto, M., & Sugane, K. (2008). Exposure to Bisphenol A Prenatally or in Adulthood Promotes TH2 Cytokine Production Associated with Reduction of CD4+CD25+ Regulatory T Cells. *Environmental Health Perspectives, 116*(4), 514-519.

Idriss, H. T., & Naismith, J. H. (2000). TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microscopy Research And Technique,* 50(3), 184-195.

Karvonen, M., Viik-Kajander, M., Moltchanova, E., Libman, I., LaPorte, R., & Tuomilehto, J. (2000). Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group. *Diabetes Care*, *23*(10), 1516-1526.

Kawai, K., Nozaki, T., Nishikata, H., Aou, S., Takii, M., & Kubo, C. (2003). Aggressive Behavior and Serum Testosterone Concentration during the Maturation Process of Male Mice: The Effects of Fetal Exposure to Bisphenol A. *Environmental Health Perspectives,* (2). 175.

Kharrazian, D. (2014). The Potential Roles of Bisphenol A (BPA) Pathogenesis in Autoimmunity. *Autoimmune Diseases*, *2014*743616. doi:10.1155/2014/743616

Kikutani, H., & Makino, S. (1992). The murine autoimmune diabetes model: NOD and related strains. *Advances In Immunology*, *51*285-322.

Krementsov, D. N., Katchy, A., Case, L. K., Carr, F. E., Davis, B., Williams, C., & Teuscher, C. (2013). Studies in Experimental Autoimmune Encephalomyelitis Do Not Support Developmental Bisphenol A Exposure as an Environmental Factor in Increasing Multiple Sclerosis Risk. *Toxicological Sciences*; doi:10.1093/toxsci/kft141.

Koh, C.-H., Khim, J. S., Villeneuve, D. L., Kannan, K., & Giesy, J. P. (2006). Characterization of trace organic contaminants in marine sediment from Yeongil

Bay, Korea: 1. Instrumental analyses. *Environmental Pollution*, 142, 39–47. doi:10.1016/j.envpol.2005.09.005

Kolb-Bachofen, V., Epstein, S., Kiesel, U., & Kolb, H. (1988). Low-dose streptozotocin induced diabetes in mice: Electron microscopy reveals single- cell insulitis before diabetes onset. *Diabetes* 37,21-27.

Lee, M. H., Lee, W. H., Todorov, I., & Liu C. P. (2010). CD4+CD25+ regulatory T cells prevent type 1 diabetes proceded by dendritic cell-dominant invasive insulitis by affecting chemotaxis and local invasiveness of dendritic cells. *Journal Immunology* 185:2493-2501. doi: 10.4049/jimmunol.1001036.

Maahs, D. M., West, N. A., Lawrence, J. M., & Davis, E. J. M (2010). Epidemiology of type 1 Diabetes. NCBI, 481-497.

Makino, S., Kunimoto, K., Muraoka, Y., Mizushima, Y., Katagiri, K., & Tochino, Y. (1980). Breeding of a non-obese, diabetic strain of mice. *Experimental Animals,* 29(1), 1-13.

Markey, C., Luque, E., De Toro, M., Sonnenschein, C., & Soto, A. (2001). In utero exposure to bisphenol A alters the development and tissue organization of the mouse mammary gland. *Biology Of Reproduction*, *65*(4), 1215-1223.

Murray, T. J., Maffini, M. V., Ucci, A. A., Sonnenschein, C., & Soto, A. M. (2007). Induction of mammary gland ductal hyperplasias and carcinoma in situ following fetal bisphenol A exposure. *Reproductive Toxicology*, *23*(Fetal Basis of Adult Disease), 383-390. doi:10.1016/j.reprotox.2006.10.002

National Diabetes Report. (2014). Retrieved from https://www.cdc.gov/diabetes/pdfs/data/2014-report-generalinformation.pdf

National Institute of Environmental Health Sciences. (2014, August 28). Retrieved from Bisphenol A: http://www.niehs.nih.gov/health/topics/agents/sya-bpa/

National Institute of Allergies and Infectious Disease. (2016). Retrieved from https://www.niaid.nih.gov/research/immune-system-research

Nikaido, Y., Yoshizawa, K., Danbara, N., Tsujita-Kyutoku, M., Yuri, T., Uehara, N., & Tsubura, A. (2004). Effects of maternal xenoestrogen exposure on development of the reproductive tract and mammary gland in female CD-1 mouse offspring. *Reproductive Toxicology, 18*803-811. doi:10.1016/j.reprotox.2004.05.002

O'Brien, B., Harmon, B., Cameron, D., & Allan, D. (1996). Beta-cell apoptosis is responsible for the development of iddm in the multiple low-dose streptozotocin model. *Journal Of Pathology, 178*(2), 176-181. doi:10.1002/(SICI)1096-9896(199602)178:2<176::AID-PATH433>3.0.CO;2-8

Paik, S., Michelis, M., Kim, Y., & Shin, S. (1982). Induction of insulin-dependent diabetes by streptozotocin. Inhibition by estrogens and potentiation by androgens. *Diabetes*, 31(8), 724-729.

Rich, S. S., Akolkar, B., Concannon, P., Erlich, H., Hilner, J. E., Julier, C., & ... Todd, J. A. (2009). Overview of the Type I Diabetes Genetics Consortium. *Genes & Immunity*, *10*S1-S4. doi:10.1038/gene.2009.84

Richter, C. A., Birnbaum, L. S., Farabollini, F., Newbold, R. R., Rubin, B. S., Talsness, C. E., & ... vom Saal, F. S. (2007). Review: In vivo effects of bisphenol A in

laboratory rodent studies. *Reproductive Toxicology*, *24*199-224. doi:10.1016/j.reprotox.2007.06.004

Roep, B. O., & Peakman, M. (2012). Antigen targets of type 1 diabetes autoimmunity. *Cold Spring Harbor Perspectives In Medicine*, *2*(4), a007781. doi:10.1101/cshperspect.a007781

Rubin, B. S., Murray, M. K., Damassa, D. A., King, J. C., & Soto, A. M. (2001). Perinatal Exposure to Low Doses of Bisphenol A Affects Body Weight, Patterns of Estrous Cyclicity, and Plasma LH Levels. *Environmental Health Perspectives*, (7). 675.

Sakaguchi, S. (2000). Regulatory T cells: Key controllers of immunologic self-tolerance. *Cell*, *101*(5), 455.

Sakowicz-Burkiewicz, M., Kocbuch, K., Grden, M., Szutowicz, A., & Pawelczyk, T. (2006). Diabetes-induced decrease of adenosine kinase expression impairs the proliferation potential of diabetic rat T lymphocytes. *Immunology*, 118(3), 402-412. doi:10.1111/j.1365-2567.2006.02380.x

Sawai, C., Anderson, K., & Walser-Kuntz, D (2003). Effect of bisphenol A on murine immune function: Modification of interferon-ϑ, IgG2a, and disease symptoms in NZB x NZW F1 mice. *Enviornmental Health Perspectives* 111(16),1883-1887.

Shafrir, E. (2007). Animal models of diabetes, Second Edition: Frontiers in research. Dr. Eleaza Shafrir (ED.) ISBN: 9780849395345.

Shin, Y., Yamaki, K., Xiaojuan, L., Tao, S., Yanagisawa, R., Takano, H., & ... Mori, Y. (2004). Prenatal exposure to bisphenol A up-regulates immune responses, including

T helper 1 and T helper 2 responses, in mice. *Immunology*, *112*(3), 489-495. doi:10.1111/j.1365-2567.2004.01900.x

Silver, M. K., O'Neill, M. S., Sowers, M. R., & Park, S. K. (2011). Urinary Bisphenol A and Type-2 Diabetes in U.S. Adults: Data from NHANES 2003-2008. *Plos ONE*, *6*(10), 1-9. doi:10.1371/journal.pone.0026868

Stachel, B., Ehrhorn, U., Heemken, O., Lepom, P., Reincke, H., Sawal, G., & Theobald, N. (2003). Xenoestrogens in the River Elbe and its tributaries. Environmental Pollution, 124497-507. doi:10.1016/S0269-7491(02)00483-9

Stassen, M., Jonuleit, H., Muller, C., Klein, M., Richter, C., Bopp, T., Schmitt, H., & Schmitt, E. (2004). Differential regulatory capacity of CD25+ T regulatory cells and pre-activated CD25+ regulatory cells on developmental, functional activation, and proliferation of Th2 cells. *Journal Immunology* 173,267-274. doi: 10.4049/jimmunol.173.1.267

Tao, B., Pietropaolo, M., Atkinson, M., Schatz, D., & Taylor, D. (2010). Estimating the Cost of Type 1 Diabetes in the U.S.: A Propensity Score Matching Method. *Plos ONE*, *5*(7), 1-11. doi:10.1371/journal.pone.0011501

Taylor, K. W. (2013). Evaluation of the Association between Persistent Organic Pollutants (POPs) and Diabetes in Epidemiological Studies: A National Toxicology Program Workshop Review. *Environmental Health Perspectives*.

Thien, K. (2009). Bis-phenol A: origin, fate, dilemma and the effects of increased background organics on its absorption by powdered activated carbon. *Final Thesis*, p.15.

Timms, B. G., Howdeshell, K. L., Barton, L., Bradley, S., Richter, C. A., & Vom Saal, F. S. (2005). Estrogenic chemicals in plastic and oral contraceptives disrupt development of the mouse prostate and urethra. *Proc National Academy of Science USA* 102(19),7014-7019.

United States Environmental Protection agency. (2009). Persistent Organic Pollutants: A Global Issue, a Global response.

United States National Library of Medicine. (2014). Retrieved from https://vsearch.nlm.nih.gov/vivisimo/cgi-bin/query-meta?query=autoimmune&v%3Aproject=nlm-main-website

University of Oxford. (2000). Phenotypic analysis of peripheral CD4+ CD8+ T cells in the rat.

Utuk, E. U. (2010). Animal models for studying diabetes mellitus. *Agriculture and Biology journal of North America*, ISSN: 2151-7525.

Wagner, D. (2011). The role of T cells in type 1 diabetes, type 1 diabetes pathogenesis, genetics and immunotherapy. Prof. David Wagner (Ed.), ISBN: 978-953-307-362-0, InTech, DO1: 10.5772/22040.

Watanabe, H., Numata, K., Ito, T., Matsukawa, A., & Takagi, K. (2004). Innate immune response in Th1- and Th2-dominant mouse strains. *Shock*, 22(5), 460-466. doi:10.1097/01.shk.0000142249.08135.e9

Wetherill, Y., Hess-Wilson, J., Comstock, C., Shah, S., Knudsen, K., Buncher, C., & ... Babcock, G. (2006). Bisphenol A facilitates bypass of androgen ablation therapy in

prostate cancer. *Molecular Cancer Therapeutics*, *5*(12), 3181-3190. doi:10.1158/1535-7163.MCT-06-0272

Wildner, G., & Kaufmann, U. (2013). Review: What causes relapses of autoimmune diseases? The etiological role of autoreactive T cells. Autoimmunity Reviews, 121070-1075. doi:10.1016/j.autrev.2013.04.001

Xu, X., Xie, L., Hong, X., Ruan, Q., Lu, H., Zhang, Q., Zhang, G., & Liu, X. (2013). Perinatal exposure to Bisphenol-A inhibits synaptogenesis and affects the synaptic morphological development in offspring male mice. *Chemosphere, vol. 91, no. 8, pp. 1073-1081.*

Yoshino, S., Yamaki, K., Li, X., Sai, T., Yanagisawa, R., Takano, H., & Hayashi, H. (2004). Prenatal exposure to bisphenol A up-regulates immune responses, including T helper 1 and T helper 2 responses, in mice. Immunology, 112(3), 489-495. doi:10.1111/j.1365-2567.2004.01900.x