5-2015

Abatement of Type 1 Diabetes as a Result of Polychlorinated Biphenyl (PCB)-153 Exposure in the Non-Obese Diabetic (NOD) Mouse Model

Jordan R. Kuiper
St. Cloud State University

ABATEMENT OF TYPE 1 DIABETES AS A RESULT OF POLYCHLORINATED BIPHENYL (PCB)-153 EXPOSURE IN THE NON-OBESE DIABETIC (NOD) MOUSE MODEL

by

Jordan R. Kuiper

B.E.S., St. Cloud State University, St. Cloud, 2012

A Thesis

Submitted to the Graduate Faculty

of

St. Cloud State University

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

St. Cloud, Minnesota

May, 2015

This thesis submitted by Jordan R. Kuiper in partial fulfillment of the requirements for the Degree of Master of Science at St. Cloud State University is hereby approved by the final evaluation committee.

Chairperson

Patricia Hughes
Dean
School of Graduate Studies

ABATEMENT OF TYPE 1 DIABETES AS A RESULT OF POLYCHLORINATED BIPHENYL (PCB)-153 EXPOSURE IN THE NON-OBESE DIABETIC (NOD) MOUSE MODEL

Jordan R. Kuiper

Type 1 diabetes (T1D) is an autoimmune disorder characterized by the T-cell- mediated destruction of insulin-producing β-cells in the pancreatic islets of Langerhans. The steady increase in prevalence and incidence of T1D across the globe suggests disease onset may be contributed by various environmental factors besides genetics, such as persistent organic pollutants (POP). Given that polychlorinated biphenyl (PCB)-153 is a highly abundant POP in both the environment and mammalian tissues, there is reason to believe the compound may be an environmental factor influencing disease susceptibility and onset. Non-obese diabetic (NOD) mice, the best experimental model for studying T1D, were exposed to intraperitoneal injections of PCB-153 in a 10-day acute (50mg/Kg or 0.5mg/Kg) or 16-week chronic (12.5mg/Kg or 0.125mg/Kg) fashion. Analysis of various immune parameters, including T-cell types and subtypes, T-cell proliferative responses, as well as their cytokine secretions, revealed that both acute and chronic exposure to PCB-153 caused significant immunosuppression in all PCB-153-exposed mice. Based on the significant decreases in $CD4^+$ T-helper cells and reduced secretion of interleukin (IL)-2 it is plausible to believe that T-helper 1 (T_{H1}) cells are the most susceptible cell population to PCB-153 exposure. This is further supported by the decrease in T1D incidence observed in mice chronically exposed to either dose of PCB-153. Overall, this study not only reveals for the first time the protective effects of PCB-153 exposure on T1D, but brings the awareness about PCB-153's "antidiabetogenic" immunossupressive effects in the context of influencing the action of other co-pollutants, as PCB-153 might mask other pollutants' effects on T1D development.

May, 2015
Month Year

Approved by Research Committee:

Marina Cetkovic-Cvrlje Chairperson

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

Chapter I

BACKGROUND

PCB-153: Chemical Structure, Properties, and Significance

Polychlorinated Biphenyl-153 follows the general chemical structure of other PCB congeners, however, this compound is unique because it is halogenated with chlorine atoms at the 2, 2', 4, 4', 5, 5' carbons (Figure 1). Due to this halogenation, PCB-153 has a high heat capacity, low conductance, and are highly soluble in oils, fats, and organic solvents (like Dimethyl Sulfoxide) (EMEP/CORINAIR, 2005). PCBs are further distinguished from one another by being either dioxin-like or non-dioxin-like, within which they can either be coplanar or non-coplanar. PCB-153 is described as being a non-dioxin-like, non-coplanar PCB (Liu *et al.*, 2014; Zhang *et al.*, 2014).

PCB-153 was used, during its production, for many different avenues including both closed and open-end applications. Examples of closed applications, includes: capacitors and transformers, heat transfer, and hydraulic fluids; whereas open-end applications includes: flame retardants, inks, adhesives, dyes, paints, pesticides, and metal coatings (Koplan, 2000).

The reason why investigation of PCB-153 is so paramount stems from the above statement. PCB-153 has been found on nearly every continent on earth; but, this alone is not the most disturbing fact. PCB levels have been shown to be higher in concentration in indoor structures, as opposed to outdoors, especially in areas with a known contamination, or which were built in the 1960s and early 1970s (before the United States' total ban on PCB usage in 1979) (EPA, 2012). Today, no imports/exports of PCB-containing products are allowed in the United States, unless granted specific access by the Environmental Protection Agency (EPA).

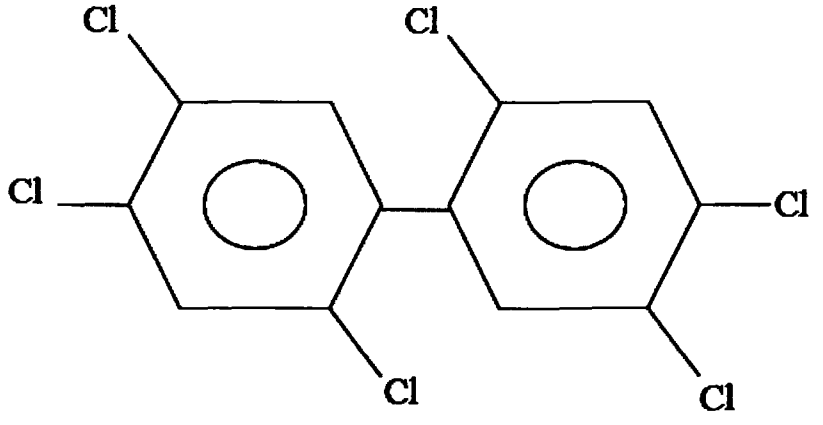

Figure 1. Chemical structure of polychlorinated biphenyl-153.

PCB-153: Biochemical Interactions

PCB-153 has been studied in various disease pathologies; however, the exact mechanisms behind this compound's interactions, are still not well understood.

Many dioxin-like PCBs are processed in the body through aryl hydrocarbon receptors (AhRs). In this instance, the compound binds to the AhR, causing the receptor chaperones to dissociate, leading the AhR to translocate to the nucleus, where it can bind to DNA and act as a transcriptional factor, bringing about changes in biological functions (e.g., xenobiotic-metabolizing enzyme cytochrome P450scc) (Denison *et al.*, 2002). Non-dioxin-like PCBs, however, have been shown to act independently of the AhR. Though the exact mechanisms behind PCB-153 processing in the body are not well understood, several studies have recently implicated that it may interact through the constitutive androstane receptor and/or pregnane-X receptor pathway(s).

The constitutive androstane receptor (CAR) pathway has recently been identified as a main pathway for recognition and metabolism of xenobiotics (compounds which are foreign to the organismal body), with receptor locations found primarily in the liver and kidney (though small amounts of receptors have been identified in the human brain and murine gastrointestinal tract) (Timsit and Negishi, 2007). The main mechanism of action for this pathway is relatively simple: a xenobiotic (e.g., PCB-153) binds to the receptor, causing the protein to translocate to the nucleus, where it can bind to DNA either causing upregulation or downregulation of target genes, with products which will further metabolize the xenobiotic (Baes *et al.*, 1994).

The pregnane-X receptor (PXR) is very similar to CAR (both are from the same nuclear receptor subfamily (NR1|2)), and is responsible for upregulation of

gene expression in which oxidative enzymes are resultant. PXR primarily forms a heterodimer with the retinoid-X receptor (RXR), and often upregulates *CYP3A4*, a gene responsible for the production of phase I oxidative enzymes (capable of metabolizing many xenobiotics) (Lehmann *et al.*, 1998).

Health Effects of PCB-153

Polychlorinated biphenyls, like PCB-153, have been associated with many disease pathologies, including: neurotoxicity/behavioral composition, liver dysfunction and carcinogenesis, and endocrine disruption.

Neurotoxicity: Discovery of the neurotoxic and neurobehavioral effects associated with PCBs were first performed back in the 1970s. One of the landmark studies to implicate these health effects as a result of PCB exposure (using Aroclor trade-name mixtures of PCBs; described later), was performed in 1972 by Dr. Joel Bitman. In this study, rats and Japanese quail were administered with varying doses of manufacturer-purchasable mixtures of PCBs (Aroclor brand, purchasable from Monsanto Company), and subsequently studied to investigate changes in biological function. Among the many effects shown in this study, sleeping times in high-dose treated subjects were significantly decreased, compared to the control groups (Bitman *et al.*, 1972). It was also found in this study that level of chlorination for PCBs had a direct effect on the level of biological response observed in the subjects. From this, most of the studies involving PCBs and biological function (or lack thereof) have been focused on only particular congeners of PCBs, or comparing dioxin-like and non-dioxin-like congeners with each other.

It has been shown that non-dioxin-like, non-coplanar congeners of PCBs (like PCB-153) are capable of inducing cell apoptosis (cell death), in a time-dose-dependent relationship, and with less vigor than the dioxin-like, coplanar congeners (Hwang *et al.*, 2001; Sanchez-Alonso *et al.*, 2003; Shin *et al.*, 2000). This apoptotic instigation is further contributed to PCB-153's (and similar congeners) ability to increase phospholipase A_2 (PLA) (and its associated Ca^{2+} influx) along with protein kinase C (PKC), in neuronal cells. This activation leads to induction of a Caspase cascade, which is one of the hallmark pathways for apoptosis instigation (Robertson and Orrenius, 2000).

Another study determined a relationship between PCB-153 exposure and neurobehavioral modifications (Johansen *et al.*, 2011). It was found that male Wistar Kyoto rats (a rodent model for Attention Deficit Disorder), treated orally with human-relevant doses of PCB-153 (using breast milk exposure levels as a baseline), had significant reductions in: brain activity, activity motivation, and decreased motor-skills.

One of the most recent studies involving PCB-153 exposure and neurotoxicity and/or neurobehavioral effects, sought to identify receptors and molecular pathways involved in brain vascular toxicity of PCB-153, in a murine model (Zhang *et al.*, 2013). In this study, it was found that low-dose internal carotid artery (ICA) infusions of PCB-153 were able to initiate inflammatory responses in the brain via toll-like receptor 4 (TLR4), which further caused silencing of tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6),

allowing a PCB-153 induced overproduction of inflammatory mediators (Zhang *et al.*, 2013).

Liver dysfunction and Carcinogenesis: Many studies have been performed to investigate the effects of PCB exposure on the function of the liver and hepatocytes. The first study to fully show the differential effects between non-dioxin-like and dioxin-like PCBs and liver function, was performed in 1983 (Parkinson *et al.*, 1983). In this study, it was shown that male Long-Evans rats treated with low-dose PCB-153 were able to induce cytochromes P450a-P450e in the liver, giving insight to the pollutant's metabolism in the liver.

Administration of PCB-153 intraperitoneally (i.p.) to female Sprague-Dawley rats was shown to increase hepatic tumor-promoting transcription factors NF-κβ and AP-1, decrease STAT3 and STAT5 (signal transducers and activators of transcription), and increase the growth rate of normal hepatocytes, in both low- and high-doses (Lu *et al.*, 2004; Tharappel *et al.*, 2002). In a related study, it was found that male B6129SF2/J mice treated with PCB-153 in a low-moderate dose, long-term had increased hepatic tumor mutations in the *Catnb* gene, particularly affecting glutamine synthetase (GS) overexpression, leading to increased tumor promotion and growth (Strathmann *et al.*, 2006).

Endocrine disruption: PCB-153 has been shown to act as an estrogenic xenobiotic in both human and murine subjects, which decreases thyroxine (T4) and triiodothyronine (T3) concentrations in male Sprague Dawley rats (Kobayashi *et al.*, 2009; Li *et al.*, 1994). Many studies have also shown delayed onset of puberty,

altered luteinizing hormone (LH) and testosterone concentrations, as well as decreased sperm counts and DNA-damage in sperms, as a result of low-dose PCB-153 exposure in rodents and goats (Dickerson *et al.*, 2011; Fusani *et al.*, 2007) . In humans, it was found that PCB exposure (either *in utero* or postnatal) led to: lowered LH and testosterone concentrations, shorter penile lengths in boys at time of puberty, delayed genital development, and pubic hair growth (Den Hond *et al.*, 2002; Guo *et al.*,2004).

Immune System: Headways in the understanding of PCB involvement with the immune system have been made possible by both epidemiologic studies and animal model experiments. In Dutch preschool-aged children, an increased incidence of infections and decreased allergic reactions associated with increased T-lymphocyte populations in blood, was observed (Weisglas-Kuperus *et al.*, 2000). However, negative associations between PCB-153, specifically, and certain T-lymphocytes were observed in a study involving Swedish infants (Glynn *et al.*, 2008). In a study using the National Health and Nutrition Examination Survey (NHANES), it was found that self-reported rheumatoid arthritis cases were increased in women with above average non-dioxin-like PCB concentrations in sera (Lee *et al.*, 2007).

An array of experimental findings is evident in literature using animal models. Fournier *et al.*, 2000 found a decrease in humoral immunity in C57BL/6 mice fed a diet rich in PCBs associated with a slight decrease in $CD8^+$ and $CD45^+$ splenocytes. Similarly, thymic and splenic atrophy was observed in marine

porpoises exposed environmentally to PCBs, as made apparent by reduced T- and B-cell counts in peripheral blood (Beineke *et al.,* 2005). Reduced T- and B-cell mitogen-induced proliferation was also observed in *L. macrochirus* (Bluegill Sunfish), *in vitro* (Duffy-Whritenour *et al.*, 2010). Overall, these studies suggest a possible immunosuppressive effect on certain immune cell subsets, based on the route and extent of PCB exposures.

Epidemiologic Studies

Several case studies have been performed in the past 30 years to better understand and correlate exposures to PCBs and incidence of health adversities, including those in the United States (Silverstone *et al.*, 2012; Sjödin *et al.*, 2014), Japan (Kanagawa *et al.,* 2008), Taiwan (Wang *et al.*, 2008), and Sweden (Rignell-Hydbom *et al.*, 2010). A few of the most influential case studies are detailed below.

United States: Two major epidemiologic studies were performed in the United States; one specifically focusing on Texas-native children and the other on factory workers involved with a PCB-related exposure in Alabama. The Texas study analyzed the blood sera collected from 300 male and female juveniles (ages 0 months-13 years), and tested for presence of PCBs (amongst other organic pollutants). It was found that levels of PCB-153 in the subjects were elevated above the reported national average (especially in the 0-2 year old and 10-13 year old categories) (Sjödin *et al.*, 2014).

The Alabama study is far more critical, since it has strong relationships with other epidemiologic studies, and has far-reaching implications. PCBs were produced in Alabama, USA, from 1929-1971 in a plant operated by Monsanto Corporation. During litigation against Monsanto, it was determined that Anniston, Alabama (the town majorly affected by the plant) was among the most heavily polluted regions in the world (with respect to soil, sediment, and air PCB-153 contamination) (Koplan, 2000). In a 2012 study, 3,320 households were randomly selected in Anniston, and blood samples were collected from each resident. A higher incidence of diabetes development was determined (though type was not provided), compared to the national average (as stated by the National Health and Nutrition Examination Survey-NHANES), with individuals having the highest average PCB congener levels in sera (7.71ppb) also having the highest rate of diabetes incidence. It was also shown that females were most likely to develop diabetes from PCB exposure (after accounting for preexisting diabetes risk factors), with non-white females being the most susceptible population (Silverstone *et al.*, 2012). However, the measurement of contaminant in the sera was a cumulative PCB concentration, and not PCB-153 specific. Given the highly variable nature of the PCB congeners, it is unclear whether the increase in diabetes incidence was directly affected by increased PCB-153 concentrations, since this unique measurement was not performed.

Japan: In 1968, a mass-poisoning event occurred in Japan colloquially called "Yūsho", which was the result of contaminated rice oil in food staples. A

nearly 30-year follow-up study on the individuals associated with this poisoning have discovered positive correlations between PCB levels in sera, age of individuals, and onset of various diseases. Many of the health adversities and clinical diagnostic biomarkers (for associated diseases) included: acneform eruptions, black comedones, cutaneous and mucosal pigmentation, hypersecretion of melbomian glands, general fatigue, headaches, cough, sputum, gastrointestinal abnormalities, increased serum γ-glutamyl transpeptidase (GTP), and opthalmological adversities. It was also concluded that adult women were the individuals most likely to have/develop the above health-related issues, when compared to all other exposed individuals (Kanagawa *et al.*, 2008).

Taiwan: Following the same mass-food poisoning event associated with PCB-contaminated rice-bran oil in the late 1970s (Yūsho), similar patterns of disease were seen in affected individuals in Tawain (where the poisoning is known as "Yu-Cheng). In 30-year follow up study, sera concentration levels of PCBs were determined for individuals affected by Yu-Cheng poisoning, and correlations with health adversities were observed. From this study, it was determined that women in the Yu-Cheng poisoning cohort were twice as likely to be diagnosed with type 2 diabetes (T2D), compared to the reference group (Wang *et al.*,2008).

Sweden: A nested-case control study performed using 150 children with type 1 diabetes and matched controls sought to correlate PCB-153 and another POP, DDT metabolite DDE, contaminations with type 1 diabetes incidence. Though the results of individual pollutant contamination were insignificant,

researchers found that co-contamination of the two compounds in higher percentiles actually had a protective effect against disease onset (Rignell-Hydbom *et al.*, 2010).

Based on the plethora of research investigating the effects of PCBs on disease pathogenesis and health adversities, it is clear that PCBs (especially PCB-153) are likely to play a role in development of inflammatory-induced diseases, including diabetes.

The Immune System

All foreign particles which come into contact with an organism, whether they be pathogenic or non-pathogenic, likely invoke the immune system in some fashion. Often perceived as the body's main defense mechanism against infection by pathogens, or initiator of an allergic response, the immune system is a key component in regulation of normal organismal homeostasis. Achievement of this homeostasis is due to a complete orchestration between two main branches of the immune system: innate immunity and adaptive immunity.

The innate immune system is the body's ancestral immune system. When an invading pathogen or microbe infiltrates the body, it is the innate immune system which mounts a response, characterized by: phagocytosis of invading pathogens, evocation of an inflammatory response by secretion of pro-inflammatory cytokines, and initiation and recruitment of other various immune cells, to quell invasion by the foreign invader. Major cells of the innate immunity

include: natural killer (NK) cells and phagocytes (macrophages, neutrophils, and dendritic cells).

Natural killer cells (NK), are important for the immune system due to their cytotoxic capabilities (mainly to virus-infected or tumorogenic cells); but, are important also for their ability to interact with other cell types, including antigen-presenting cells, as well as T-cells. The main cell surface marker distinguishing NK cells is NK1.1 surface marker.

Macrophages are the descendants of the monocyte lineage, and are the primary phagocytes of the immune system. They engulf apoptotic cells as well as pathogens, all while producing effector molecules, to help mount the immune inflammatory response. They also play a key role in adaptive immunity (discussed later) in the sense that they present antigens to adaptive immune cells, priming them for their immune-related actions. The major cell surface maker of macrophages is CD11b.

Neutrophils are the most abundant white blood cell subset present in mammals, even though they have a relatively short lifespan. They are the first responders to the site of an infection (during the acute inflammatory phase), and are the primary bulk of wound/lesion pus. They mainly kill invading pathogens and infected cells by formation of phagosomes and use of reactive oxygen species, leading to cell respiratory burst.

Dendritic cells are the last major phagocyte of the immune system, are antigen-presenting cells (APCs); they are capable of inducing a primary immune

response in resting naïve T- cells (discussed later). Also, they are able to control B-cell function and are important in development of immunological memory.

The adaptive immune system, is an *acquired* immunity, and is antigen-specific (unlike innate immunity). This branch of the immune system, too, contains two main subsets: humoral immunity and cell-mediated immunity.

Humoral immunity, or antibody-mediated immunity, is associated mostly with B-cells, and the antibodies they secrete.

B-cells, descendants of hematopoietic stem cells, establish antigen receptors (surface immunoglobulins or antibodies) during differentiation in the bone marrow. Two major types of B-cells exist: memory B-cells (which are capable of initiating a secondary immune response when an organism is challenged by the same pathogen) and plasma cells (which are the major effector B-cells and are responsible for antibody production).

Cell-mediated adaptive immunity, or T-cell mediated immunity, involves the activation of T-cells, and production of cytokines for further immune response amplification. The main cells of this type of adaptive immunity includes: T-helper (T_H), T-cytotoxic (T_C), and T-regulatory (T_{REG}) cells. Many other subsets exist, though they are exceedingly minute.

T-helper cells are a uniquely diverse set of immune cells, which can be further segregated into subsets T_{H1}, T_{H2}, and T_{H17}. All T-helper cells are CD4+/CD8- effector cells; however, T_{H1} cells are those which are responsible for activating and stimulating both T-cytotoxic cells and macrophages. As part of this

activation, they secrete cytokines IL-2 and IFN-γ. T_{H2} cells, like T_{H1} cells, regulate the actions of many other cell types. More specifically, T_{H2} cells can regulate the function of antigen-specific B-cells (which produce antibodies), mast cells, and eosinophils. By releasing IL-10, T_{H2} cells are able to down-regulate the production of IFN-γ in T_{H1} cells (though production of IFN-γ by T_{H1} cells can actually inhibit proliferation of T_{H2} cells). T_{H2} cells also secrete IL-4, which creates a positive feedback loop with T_{H2} cells, further stimulating T_{H2} cell production. T_{H17} cells, one of the most recently discovered T-cell subsets, has profound anti-microbial and anti-fungal properties at the epithelial and mucosal barriers. The primary cytokine secreted by T_{H17} cells is IL-17a.

T-cytotoxic cells are $CD4^-CD8^+$ T-cells, which are capable of directly killing infected and/or damaged somatic cells of the body. Upon exposure to the target cell(s), T_C cells can kill in two major pathways: perforin/granzyme pathway, triggering caspase-dependent apoptosis; or, Fas-ligand-dependent apoptosis. The major cytokines secreted by these cells include: IFN-γ, TNF-α, and TNF-β.

T-regulatory cells are a small but powerful subset of T-cells. T_{REG} cells are a subset of T-cells which are the primary regulatory agents of the immune system expressing surface marker $CD4^+CD25^+$. CD25 is the α-chain of the IL-2 receptor. However, recent studies have made them more commonly known for their expression of the transcriptional regulator, FoxP3, a necessary component for T-regulatory cell differentiation. T_{REGS} are autoreactive T-cells which do not die via negative selection; rather, they escape death (by largely unknown circumstances)

and seek to patrol the body for unbalanced autoimmune reactions (Sakaguchi, 2000). The primary cytokines secreted by this cell type are: IL-10 and TGF-β.

All cells of the immune system play an important role in organismal homeostasis, no matter how small the cell population; when any one of the cell populations loses its regular function, health adversities become apparent.

Diabetes

Diabetes is a blanket term for many different conditions that result with increased glucose levels in the blood (hyperglycemia) with consequent excessive thirst and urine excretion. The most common forms of diabetes are: adult-onset diabetes (type 2 diabetes) and juvenile diabetes (type 1 diabetes).

Type 2 diabetes (T2D) is often diagnosed in adults and in close association with the risk factors such as obesity and cardiovascular disease (Iqbal, 2007). This form of disease is non-HLA-gene related (the primary gene responsible for type 1 diabetes susceptibility), and is further characterized by varying degrees of insulin resistance and β-cell death (Cnop *et al.*, 2005; Park *et al.*, 2005). A secondary subset of T2D has been identified and is now known as latent autoimmune diabetes in adults (LADA). The prevalence of this subset is 10% in T2D diagnosed patients between 40 and 75 years of age, with a similar rate found in non-insulin dependent patients above the age of 35 years. This subset is often diagnosed as type 2 diabetes, however, further studies have shown that this form of diabetes is primarily auto-antibody dependent, suggesting it has further autoimmune actions comparable to that of type 1 diabetes (Stenstrom *et al.*, 2005).

Type 1 Diabetes in Humans

Type 1 diabetes (T1D) in humans is characterized by the progressive destruction of insulin-producing β-cells in the pancreatic islets of Langerhans. This destruction of β-cells is instigated by infiltrating mononuclear cells (T-cells and macrophages). A histological lesion called insulitis, resulting in a sequential decrease in β-cell mass. Insulitis is initiated by exposure of pancreatic β-cells' autoantigens that instigate an autoimmune response. Some of the confirmed autoantigens capable of inaugurating insulitis in humans are: glutamic-acid decarboxylase (GAD)-65 and -67, insulinoma antigen 2 (IA-2), and insulin. Under normal conditions, immune cells would recognize these antigens as "self" antigens, and not mount an immune response; however, in a T1D patient, the immune cells fail to discriminate "self" antigens from "foreign" antigens, and commence an onslaught against its own cells (Notkins, 2002). T1D is considered a T-cell mediated disease, and therefore it is important to understand each T-cell's individual role in disease pathogenesis (Figure 2).

T-helper 1 (T_{H1}) cells are considered pathogenic in T1D, due to their inhibition of T-helper 2 cell differentiation by IFN-γ. IFN-γ is important for immune cell trafficking, and acts with TNF-α, IL-1β, and IL-2 to potentiate disease (Wagner, 2011).

T-helper 2 (T_{H2}) cells are considered protective in T1D, due to their inhibition of T-helper 1 cell differentiation by IL-4. IL-4 is important in anti-

inflammatory immune responses, along with IL-10, countering the effects of pathogenic cell subsets and their cytokines (Wagner, 2011).

T-helper 17 (T_{H17}) cells are considered pathogenic in T1D (though this is still widely debated), and have been linked with several other autoimmune disorders. The pathogenic effects of T-helper 17 cells are mainly caused by secretion of IL-17 and IL-22, both of which have distinct anti-microbial properties (Wagner, 2011).

T-regulatory (T_{REG}) cells are thought to be protective in T1D by creating a balance between pathogenic and protective immune cell responses. T-regulatory cells have been shown to limit the production of IL-2, a cytokine which is necessary for adequate T-cell proliferation, differentiation, and growth (Smith, 1988; Wagner, 2011).

T-cytotoxic cells (T_C) are pathogenic in T1D and are capable of directly killing pancreatic β-cells, by recognizing particular motifs of the insulin protein (Pinkse *et al.*, 2005).

In addition to the immune attack instigated by T-cells, macrophages aid in the killing of β-cells by secreting pathogenic cytokines IL-6 and TNF-α. In addition to the release of these cytokines, macrophages are capable of producing reactive oxygen species, further destroying β-cells of the pancreas (Jun *et al.*, 1999).

Though, all of the aforementioned immune cells are the primary effectors of T1D pathogenesis, the individual susceptibility to develop this disease is largely dependent on inherited genes. In humans, the major histocompatibility complex

(MHC) is colloquially called the HLA complex (which contains over 200 genes). The two classes of MHC molecules produced from these genes are responsible for the proper function of T-cells, in terms of their antigen recognition by aid of antigen presenting cells. HLA genes are highly polymorphic and contain several alleles; due to the high degree of variability (in terms of inheritable haplotypes), it is possible for an individual to acquire a haplotype which is either highly pathogenic or protective for T1D. The strongest genes associated with susceptibility to T1D, in humans, has been shown to be the DQ and DR class II HLA genes (Notkins, 2002).

Though, many advances have been made in the understanding of T1D development in humans, most research is performed in comparable animal models. The best animal model for the study of T1D is the non-obese diabetic (NOD) mouse.

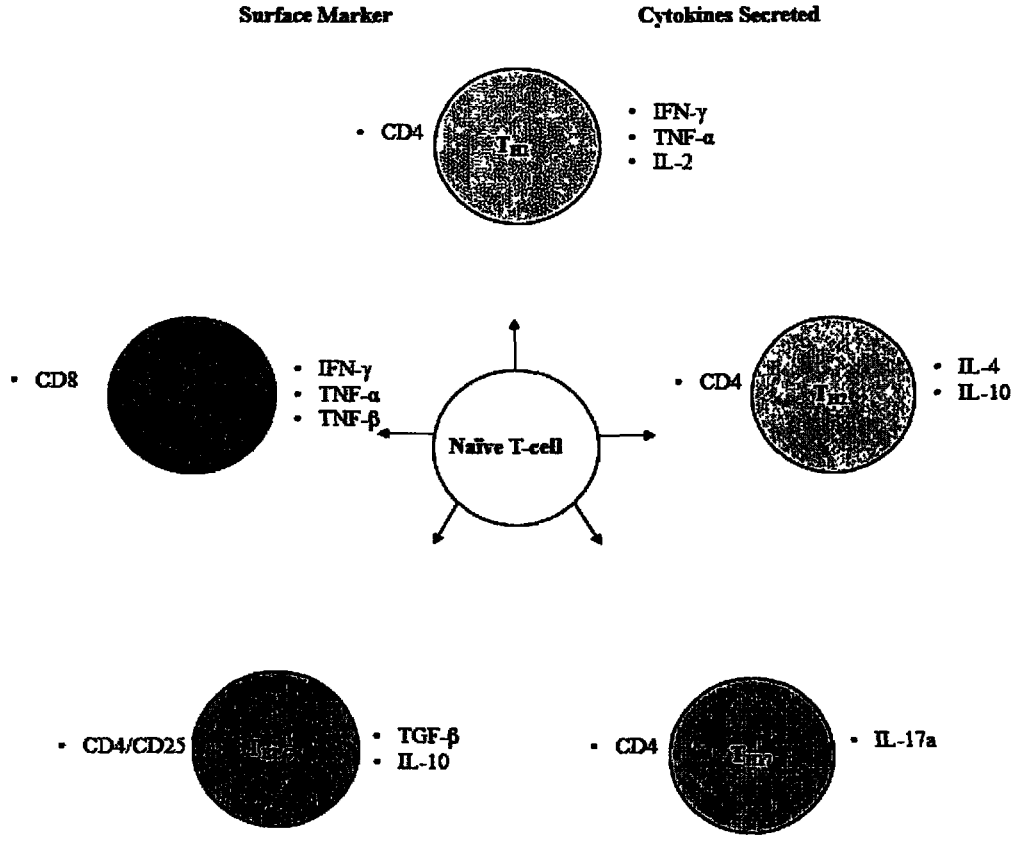

Figure 2. T-cell subsets characterized by their surface markers and particular cytokines.

NOD Mice

History of the NOD Mouse: Development of the Non-Obese Diabetic mouse strain can be traced back to 1966, when researchers at the Shionogi Laboratory began work with a mouse strain (JCl:ICR), which was an inbred strain of mouse known for spontaneously developing cataract. This strain of mouse became colloquially known as the CTS (cataracts and small eyes) mouse, which was selectively bred to retain homozygosity of these recessive traits. After the sixth

generation of breeding, researchers noticed that some mice displayed higher frequency of urination (polyuria) and urine volume, compared to others in the colony. These mice which displayed polyuria were tested for glycemia levels (as cataract is actually a common complication related to diabetes development), with the result confirming hyperglycemia (Delovitch and Singh, 1997; Shafrir, 2007).

The hyperglycemic mice were selectively bred for 10 generations, in which two lines were established: mice with euglycemia and hyperglycemia. After 13 generations of inbreeding, two strains [one with normal fasting blood glucose levels (100mg/dL) and another with high fasting blood glucose levels ($\geq$150mg/dL)] were established. At the 20th generation, a mouse in the normal line of mouse was observed to have: polyuria, polydipsia, and cachexia, suggesting that a mouse in the normal line had spontaneously developed diabetes. Inbreeding of this mouse continued to develop an inbred strain of mouse which would develop spontaneous diabetes, known now as the Non-Obese Diabetic mouse (Delovitch and Singh, 1997; Kikutani and Makino, 1992; Shafrir, 2007).

Characteristics of the NOD mouse: NOD mice, male and female, spontaneously develop T1D by infiltration of mononuclear cells into pancreatic Islets of Langerhans resulting in insulitis. Insulitis is not observed in NOD mice before three weeks of age; however, by three weeks of age, 70-90% of female mice begin development of insulitis and by 20 weeks of age, nearly 100% of both sexes of mice begin development of insulitis. Decreases in pancreatic insulin content are markedly observed in NOD females at/around 12 weeks of age and several weeks

later in males of the same colony. Diabetes is observed in mice with conjugation of blood glucose measurements at or above 220mg/dL. After insulitis is in full effect, there is a three to four week period where: cachexia, polyuria, polydipsia, hypoinsulinemia, and hyperglucagonemia occurs. It is important to note that hyperglycemic mice become ketonemic but not ketoacidotic; which relates to histological profile of NOD mice pancreata where selective destruction of β but not non-β-cell islet cells are observed (Delovitch and Singh, 1997; Kikutani and Makino, 1992; Shafrir, 2007).

Type 1 Diabetes in NOD Mice

The NOD mouse model of type 1 diabetes is similar to that of human origin (though some key differences exist, and will be identified later in this paper). The pathogenesis is classified by various stages of disease onset, including: polygenic inheritance of specific MHC class II alleles as well as several other non-MHC loci, immune cell orchestration, and insulitis lesion characterized by intra-islet inflammatory infiltration (Delovitch and Singh, 1997).

Genetic factors associated with NOD Type 1 diabetes development: Autoimmune type 1 diabetes in NOD mice, as previously stated, is largely governed by polygenic control (though various environmental triggers have strong influences on disease pathogenesis, too). Many studies have been performed using NOD mice in the hopes of identifying homologous loci for disease pathogenesis in humans; such studies have given illumination to the key genes and loci acting as causative factors in T1D. In three major studies (Kikutani and Makino, 1992;

Leiter, 1990; Leiter and Serreze, 1992) the genetic contributor most associated with T1D susceptibility in the NOD mouse was determined to be the MHC-haplotype *H-2*^{g7} on chromosome 17. This haplotype causes a lack of expression of *I-E* (homologous to DR in human genome) and expression of *I-Aβ* locus (homologous to diabetogenic *HLA-DQB* non-aspartic acid^{57}-containing alleles in humans) (Leiter, 1993).

Further studies indicate insulitis development being instigated by heterozygous or homozygous expression of *H-2*^{g7} haplotype; however, severe insulitis necessary to permit clinical phenotype of diabetes can almost always only be observed with NOD mice homozygous for the haplotype affecting MHC-class II. Also, mice homozygous for the *H-2*^{g7} haplotype lack the ability to express *I-E* molecules on their antigen presenting cells causing insufficient communication between cells of the innate and adaptive immunities (Lund *et al.,* 1990; Uehira *et al.,* 1989). Several other non-MHC associated regions of the NOD genome have been identified as contributors to disease development. These genes are now collectively and colloquially known as *Iddm* genes, with at least 20 being identified (Wicker *et al.,* 1994).

Chapter II

INTRODUCTION

Type 1 diabetes (T1D), a T-cell dependent autoimmune disease resulting in the destruction of insulin-producing β-cells of the islets of Langerhans by immune cells, is a disease afflicting nearly 3 million people in the United States (JDRF, 2014), and is rising in both prevalence and incidence in countries across the globe. In the United States alone, the prevalence of T1D increased by 21% in children (Dabelea *et al.*, 2014), with the incidence increasing by approximately 2.7% per year in the recent decade (Lawrence *et al.*, 2014).

T1D has been axiomatic to arise from autoimmune T-cell dependent destruction of insulin producing beta cells of the pancreas, a process well studied in the NOD mouse model (Anderson and Bluestone, 2005; Lu *et al.,* 2004; Schatteman *et al.*, 2000; Strathmann *et al.*, 2006). Pathogenesis of T1D in these mice is spontaneous, and occurs in a manner similar to humans. The overall pathogenesis of T1D is believed to be an orchestration between various immune cell subsets and the cytokines they emit. Particularly, the destruction of beta cells is believed to be influenced by the cytokines: interleukin (IL)-2 and interferon (INF)-γ, keystones to the T-helper 1 and T-cytotoxic responses; conversely, cytokines IL-4 and IL-10, evoked by the T-helper 2 response, are thought to be protective

entities with T1D development (Muller *et al.*, 2002; Rabinovitch, 1998). IL-17 is thought to be pathogenic in nature during T1D development, and is a hallmark to T-helper 17 (Betteli *et al.*, 2007), whereas transforming growth factor (TGF)-β and IL-10, produced by the T-regulatory cells, are thought to be protective (Grunnet and Mandrup-Poulsen, 2011). Autoimmune diseases, like T1D, have been shown to be complicit with genetic and environmental factors, both of which contribute to onset and individual susceptibility (Ahlborg *et al.*, 1992; Česko-Slovenská Pediatrie, 2007; Koplan, 2000; Krogenaes *et al.*, 1998; Strathmann *et al.*, 2006). Advances have been made with the understanding of genetics' roles in development of T1D, through use of human gene studies (Noble and Erlich, 2012; Pociot and McDermott, 2002) and by use of the non-obese diabetic (NOD) mouse (Anderson and Bluestone, 2005; Wicker *et al.*, 1994); however, the role of environmental exposures on disease onset and pathogenesis is still poorly understood.

Several paradigms regarding environmental influences on T1D development exist, such as the hygiene hypothesis and viral/bacterial infections (Knip and Simell, 2012; Okada *et al.*, 2010). Persistent organic pollutants (POPs) have emerged relatively recently on the scene. Persistent organic pollutants (POPs) are organic compounds which remain indelible in the environment, resisting photolytic, biological and chemical degradation (Ahlborg *et al.*, 1992; Krogenaes *et al.*, 1998; Longnecker and Daniels, 2001; Ritter *et al.*, 1995). Often, these compounds are halogenated, and as such, have extremely low water solubility and

high lipid solubility, allowing them to bioaccumulate in the adipose tissues of numerous organisms (Ahlborg *et al.*, 1992; Ritter *et al.,* 1995). POPs are also often semi-volatile, meaning they are able to travel long distances in the atmosphere before being deposited onto the surface of the earth (EMEP/CORINAIR, 2005). Tests have shown traces of several POPs to be present in samples taken from the Sub-Saharan desert and Antarctica (areas where use of POPs were never established) (EPA, 2009; Ritter *et al.,* 1995; WHO, 2008).

Though many different types of POPs exist in the environment, the ones of greatest concern are deemed the "Dirty Dozen", due to their mass production in the 20^{th} century, extreme resistance to degradation, and relation to adverse health effects. The term "Dirty Dozen" includes several compounds, including: DDT, hexachlorocyclohexanes (HCHs), and polychlorinated biphenyls (PCBs). Though each of the pollutants listed above represent a unique threat to both organismal health and the environment, the ones of emerging concern given their ubiquity, vastness, and relatively unknown mechanisms of action, are the polychlorinated biphenyls (EPA, 2009; WHO, 2008).

Polychlorinated biphenyls are highly unique compounds which were put into production beginning in the 1930's (EMEP/CORINAIR, 2005). PCBs are often separated by International Union of Pure and Applied Chemistry (IUPAC) nomenclature or their trade name (e.g., Aroclor) and consists of 209 different congeners (WHO, 2008). The congeners are often separated into two main groups, based on chlorination and conformation of the compounds, themselves. The PCB of

greatest biological concern, arguably, is polychlorinated biphenyl-153 (a non-dioxin-like, non-coplanar, di-*ortho*-substituted compound), the PCB found in the highest concentration in sera and lipid analysis in nearly all animal and human models tested compared to other extracted congeners (refer to Table 1). Also, PCB-153 has been attributed to being a: carcinogen, obesogen, hepatoma promoter, neurobehavioral disruptor, and endocrine disruptor (EMEP/CORINAIR, 2005; EPA, 2009; Lee *et al.,* 2006; Longnecker and Daniels, 2001; Lu *et al.,* 2004; Wojtowicz *et al.*, 2001). Speaking strictly towards the immune system, ΣPCBs have been associated with immunostimulation (Lee *et al.*, 2007; Tsai *et al.*, 2007) or immunosuppression (Duffy-Whritenour *et al.*, 2010; Guo *et* al., 2004; Tan et al., 2003; Weisglas-Kuperus *et al.*, 2000).

Most of the studies investigating effects of PCBs on the immune system are focused on cumulative (or total) PCBs (ΣPCBs), especially with regards to epidemiologic studies. In general, ΣPCBs have been associated with various effects on the immune system in both human and animal models. In humans, ΣPCBs have been associated with increased prevalence of recurrent infections and certain allergic diseases in children (Weisglas-Kuperus *et al.,* 2000). In animal models, the effects on the immune system included: decreases in splenic humoral immunity (Fournier *et* al., 2000), thymic and splenic atrophy, characterized by decreased peripheral T- and B-cell counts (Beineke *et al.*, 2005), and reduced T-and B-cell mitogen-induced proliferation, *in vitro* (Duffy-Whritenour *et al.*, 2010). Ortho-substituted PCBs, specifically, have been shown to directly kill thymocytes and

induce thymic atrophy (Tan *et al.*, 2003). With regards to autoimmunity, human studies in the context of ΣPCBs have shown increased anti-GAD antibodies in human sera, indicative of aggravation of T1D pathogenesis (Langer *et* al., 2002), and increased prevalence of rheumatoid arthritis (Lee *et al.*, 2007).

With PCB-153 alone, studies have reported contradictory findings ranging from: no effect on T_{H1}/T_{H2} cell differentiation of human white-blood cells (WBCs) *in vitro* (Gaspar-Ramirez *et al.*, 2012), decreased $CD8^+$ T-cytotoxic cells associated with decreased incidence of respiratory infections in neonates/children (Glynn *et al.*, 2008), and immunomodulation of *M. galloprovincialis* (mussels) hemocytes, particularly altering microbicidal activity and lysosomal enzyme release from hemocytes and increasing p38 and JNK mitogen activated protein kinases (MAPKs) (Canesi *et al.*, 2003).

Concerning the relationship between the POPS and T1D studied in the experimental mouse models, a few studies have investigated the influence of BPA and DDE, observing aggravation of disease in both compounds (Bodin *et al.*, 2013; Cetkovic-Cvrlje *et al.*, 2015). Whereas a plethora of published epidemiologic studies suggest a positive association between PCB exposure and type 2 diabetes (T2D) onset, exist (ATSDR, 2000; Kanagawa *et al.*, 2008; Sjödin *et al.*, 2014; Silverstone *et al.*, 2012), there is only one epidemiologic study that specifically identifies a relationship between PCBs and T1D, indicating a potential protection from disease onset concurrent with an elevation of PCB-153 and another POP, DDE (Rignell-Hydborn *et al.*, 2010). Another epidemiologic study showed a

decrease in "diabetes" onset in Taiwanese males belonging to a cohort of individuals who accidentally ingested PCBs due to rice bran oil contamination (Yu-Cheng cohort), however disease type (T1D or T2D) was not reported (Li *et al.*, 2013). However, there has not been a study performed to directly investigate the association of PCB-153 exposure and the development of T1D in an experimental mouse model.

Therefore, the aim of this study was to investigate the effects of PCB-153 exposure on T1D development in the NOD mouse model. As T1D is a T-cell-mediated disease (Cetkovic-Cvrlje *et al.*, 2003; Cetkovic-Cvrlje *et al.*, 2012), the effects of PCB-153 on T-cell function were studied *in vitro*, as well as *in vivo* during acute and chronic exposure to the compound PCB-153. Specifically, spleen cell counts, viability, T-cell proliferation, T-cell subsets (and other immune cell types), and cytokine levels were determined after the acute and during the chronic exposure to PCB-153.

Chapter III

AIMS

The ultimate purpose of this study is to investigate and illuminate the effect of a particular POP, 2, 2', 4, 4', 5, 5'-hexachlorobiphenyl-polychlorinated biphenyl-153 (PCB-153), on the immune system and diabetes development in NOD mice. Knowing that type 1 diabetes is an autoimmune, T-cell mediated disease, it was hypothesized:

1. PCB-153 will exhibit an effect on the T-cells of NOD mice
2. PCB-153 will effect type 1 diabetes development in NOD mice.

To test these hypotheses, the following experimentations were performed:

1. Effect and potential mechanism of PCB-153 action on T-cells were studied, *in vitro*, by analyzing NOD T-cell proliferation, mode of cell death after exposure to PCB-153, and dose response of T-cells exposed to varying concentrations of PCB-153.
2. Effects of PCB-153 on T-cell proliferation, cell viability, T-cell and immune cell subsets, and cytokine profiles of NOD mice with acute exposure to the compound were studied

3. Effects of PCB-153 on type 1 diabetes development, body weight composition, and glucose levels in NOD mice, exposed chronically to the compound were studied
4. Effects of PCB-153 on T-cell proliferation, cell viability, T-cell and other immune cell subsets, and cytokine profiles of NOD mice during the chronic exposure to the compound were studied.

Chapter IV

MATERIALS AND METHODS

Mice

Non-obese diabetic (NOD) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) in 2012. All mice were kept in standard cages with a 12-hour light/dark cycle and allowed to eat and drink *ad libitum* (Harlan Tekland 18% Global Protein Diet 2018 and distilled water, respectively). All experimental mice were kept in separate cages based on sex, and were chosen for experiment based on age, with the range being eight to ten weeks of age. Mice were also examined visually to ensure there were no health issues or physical ailments, both before, and then during the course of the experiments. All procedures performed on the mice used throughout the course of this project were approved by Saint Cloud State University's Institutional Animal Care and Use Committee (IACUC), prior to experiment start (project ID #5-75).

Experimental Design

In vitro experiments: To determine the effects of PCB-153 on T-cells, single cell suspensions were obtained from the spleens of healthy (not diabetic) male NOD mice. To stimulate T-cell proliferation, mitogen concanavalin A was

used in the concentration of 3μg/mL in complete media. A serial dilution of PCB-153 was prepared from 40mg/mL PCB-153 stock and then diluted in complete media, creating a range of 100μg/mL to 0.4μg/mL PCB-153. The dimethylsulfoxide (DMSO) concentration in the wells with the highest concentration of PCB-153 (100 μg/ml) was less than 2.5%, a level which is found to be not toxic to lymphocytes (Cárdenas-González *et al.*, 2013). Various controls were employed in this experiment, to ensure DMSO levels had no impact on T-cell proliferation. In these controls, cells were exposed to DMSO in the concentration comparable to that of each PCB-153 well, and were either stimulated or unstimulated by ConA. In addition to those controls, a baseline control of just cells and medium (for unstimulated) or ConA (for stimulated) were used to compare "normal" proliferation against proliferation achieved by PCB-153-exposed cells. Cells were cultured in the concentration of $4x10^6$ cells/mL in the 96-well-plate, in total volume of 100μL with addition of adequate amounts of PCB-153 and ConA. Cells were cultured for 72 hours in a growth chamber at 37°C and 5% CO_2, and proliferation of T cells was then analyzed by use of Alamar Blue colorimetric assay, as described above.

Diabetes incidence: Incidence of type 1 diabetes in 8-9-wk-old NOD female mice was followed by determination of hyperglycemia after administration of PCB-153 in a high (12.5mg/Kg)- or low (0.125mg/Kg)-dose, or vehicle control, up to 16-weeks or until diabetes was confirmed, whatever came first. For controls, DMSO was added to the corn oil vehicle in the same concentration as present in the

high-dose PCB-153 injections, to ensure no differences observed were from the diluent. All injections were administered intraperitoneally (i.p.).

Immune parameters: Determination of what immune parameters were affected by administration of PCB-153 was achieved by treating 8-9-week-old NOD males with PCB-153 in varying dosages: high-dose PCB-153 (50 mg/kg), low-dose PCB-153 (0.5 mg/kg), and a control group. All PCB-153-treated groups received PCB-153 in the corn oil; the control group received injections of corn oil vehicle with DMSO in the amount present in the high dose injections. Mice were injected on days 1, 3, 5, 7, 9, and subsequently sacrificed on day 10. Spleens were harvested, and processed for further examination, including: splenic cell counts, cell viabilities, immunophenotyping, T-cell proliferation, and cytokine profiling.

For the chronic exposure to PCB-153, 8-9-wk-old NOD female mice were chosen in order to follow the immune parameters during the development of disease. Mice were administered with two doses of PCB-153: the high-dose group received 12.5mg/Kg, and the low dose group 0.125mg/Kg injections of PCB-153 in corn oil. The control group was administered with corn oil vehicle containing the same amount of DMSO present in the high dose group injections. All injections were performed i.p. and occurred biweekly. Mice were sacrificed at three different time points, post-beginning of the PCB-153 administration: at 2 weeks, 8 weeks, and 16 weeks. Spleens were harvested, and processed for further examination, including: splenic cell counts, cell viabilities, immunophenotyping, T-cell proliferation, and cytokine profiling.

PCB-153 Preparation and Administration

PCB-153 (Ultra Scientific, North Kingstown, RI, USA; and Cerilliant Corporation, Roundrock, TX, USA) was dissolved in either 250µL or 6.25mL of DMSO at room temperature, to create a stock solution of 40mg/mL. Since PCB-153 is extremely hydrophobic, stock solution was placed into a 50Hz sonicating water bath at room temperature for five minutes, which aided in particulate dissolution, then stored at room temperature in 1.5mL amber Eppendorf tubes, or in 15mL conical tubes wrapped in aluminum foil, until ready for use.

For in vivo exposures, the acute treatment cohort received injections in the concentrations of 50mg/Kg and 0.5mg/Kg whereas, the chronic treatment cohort received injections in the concentrations of 12.5mg/Kg and 0.125mg/Kg. All drug concentrations were prepared in 1.5mL tubes, by first adding the injection vehicle (100% corn oil; Sigma Aldrich Corp., St. Louis, MO, USA), then the PCB-153 dissolved in DMSO. The mixture was vortexed at high speed to create a drug suspension. The amount of drug to be added to each 1.5mL tube was determined based on the injection volume. The literature recommendations of 6.7µL/g b.w. were followed to determine the injection volume (Ter Veld *et al.*, 2008). The basic formula is as follows:

$$\frac{\textit{Amount PCB 153 desired}}{\textit{1Kg body weight}} = \frac{\textit{Necessary amount of PCB 153 to be used in solution}}{\textit{Average body weight of all mice to be injected}};$$

by solving for the necessary amount of PCB-153 to be used in solution, this value can then be placed into the following formula:

$$\frac{40mg\ PCB\ 153}{1mL}\_\frac{Necessary\ amount\ of\ PCB\ 153\ to\ be\ used\ in\ solution}{Necessary\ amount\ of\ stock\ volume};$$

this allowed the determination of how much PCB-153 stock solution would be required, in order to make the injection solutions. For the low-dose injections, the PCB-153 stock was first diluted 100x in DMSO, in order to keep the DMSO concentration in each injection constant.

In *in vitro* experiments, PCB-153 was diluted from stock (40mg/mL) to 100μg/mL to 0.4μg/mL. PCB-153 solutions were prepared in autoclaved 1.5mL Eppendorf tubes, by first creating a 400μg/mL PCB-153 solution, then serial diluting with complete media to make lesser concentrations of PCB-153, which when added to culture results in final concentrations ranging from 100μg/mL to 0.4μg/mL.

Glucose Measurement

Tail vein blood sampling was performed by placing mice in a restrictive container, warming the tail in warm water (30°C) for 15 seconds, then using a 28g needle to prick a lateral tail vein, and take approximately 0.6μL of blood onto a glucose strip. The glucose strips were read on an Accu Check blood glucose meter (both the strips and meter were purchased from Roche Diagnostics, Indianapolis, IN, USA). Mice were bled on a weekly basis and two consecutive readings at or above 220mg/dL qualified mice as being diabetic.

Single Cell Suspension Preparation and Cell Counts/Cell Viability Determination

Single cell suspension: After mice reached the predetermined endpoints, became diabetic, or was to be used for *in vitro* analysis, they were sacrificed and spleens harvested (as described in Cetkovic-Cvrlje *et al.*, 1997). Spleens were placed onto a 70 μm nylon mesh strainer (BD Falcon, St. Jose, USA) which was pre wetted with one mL of 1X PBS, by use of autoclaved forceps. By placing the strainer on top of a 50mL conical tube, the spleen was able to be meshed with a sterile syringe plunger, collecting cells into the conical tube, but excluding the connective tissue. The cells were suspended in a total of five mL of 1X PBS, then placed into a Beckman CS-6R centrifuge, and spun at 1200 rpm for five minutes. After centrifugation, the supernatant was decanted and pellet resuspended in 750μL of ACK Lysing Buffer (NH_4Cl 8.29g/L, $KHCO_3$ 1.0g/L, EDTA $Na_2 \cdot 2H_2O$ 0.0375g/L; Lonza BioWhittaker, Walkersville, MD, USA). After one minute incubation at room temperature, the suspension volume was brought up to five mL with 1X PBS, and spun in the centrifuge again. The next three washes were performed by decanting the supernatants and resuspending cells in five mL of 1X PBS.

Cell counts and viability: In order to determine the total cell counts and cell viability present within each splenic single cell suspension, the Trypan Blue (Lonza BioWhittaker, Walkersville, MD, USA) exclusion method was performed. Splenocytes from the single cell suspensions were mixed with Trypan-Blue in the ratio of 1:20, and placed onto a hemocytometer. The cells were then viewed

immediately under a light microscope at 40X to determine the total cell counts and viability. Cells which were viable did not take up the blue dye into its cytoplasm, whereas dead cells appeared blue from taking up the dye. Viability was represented as a percentage of alive (viable) cells to the total number of cells counted.

T-cell Proliferation Assay

Ex vivo: To determine the effects PCB-153 has on T-cell function, single cell suspensions were obtained from the spleens of mice treated in the acute and chronic fashion via methods previously described (Cetkovic-Cvrlje *et al.*, 2001; Cetkovic-Cvrlje *et al.*, 2005). To stimulate T-cell proliferation, mitogen concanavalin A (Sigma, St. Louis, MO, USA) was used in the concentration of 3μg/mL in complete media [RPMI-1640 with 10% Fetal Calf Serum and 1% Penicillin and Streptomycin (1 unit/mL) (Sigma, St. Louis, MO, USA)]. Cells were cultured in the concentration of $4x10^6$ cells/mL in the total volume of 100μL in the 96-well-plate with addition of adequate amounts of ConA (for stimulated wells) or complete media (for unstimulated wells). Cells were cultured for 72 hours in a growth chamber at 37°C and 5% CO_2 Proliferation of T-cells were analyzed by use of Alamar Blue colorimetric assay. Alamar Blue colorimetric assay (Invitrogen, Grand Island, NY, USA), reduces the active component (Resazurin), which appears blue, to Resafurin, which appears red-fluorescent, by the various reduction reactions of metabolically active immune cells. The compound was added to the proliferation wells in the amount of 10μL (10% of sample volume). Once the compound was added, the assay proceeded in the same growth chamber for five to

eight hours at the end of which the plate was read in an ELISA plate reader (UniRead 800, GeneMate) at a wavelength of 570nm. Wells with sufficient T-cell proliferation appeared purple/pink in color, whereas control (complete media only) and unstimulated wells appeared blue.

Apoptosis Detection

In order to determine whether suppressive effects of PCB-153 on T-cells *in vitro* were caused by apoptosis, apoptosis detection by use of flow cytometry, was performed (with 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: 50, 12.5, or 1.6μg/mL PCB-153, in a 12-well plate. Cultures were placed in a growth chamber at 37°C and 5% CO_2 for 24 hours. After incubation, cells were collected, washed twice with 1x PBS, and $4x10^5$ cells from each sample were suspended in 100μL binding buffer, per instructions of the manufacturer. To explain the methodology briefly, cells actively undergoing apoptosis display the protein phospatidylserine (PS) on its cell surface. Annexin-V, a protein provided with the kit, binds strongly to PS, and the fact that the Annexin-V is conjugated with flourochrome FITC allows PS^+ cells to be detected by flow cytometry. In order to distinguish between apoptotic and necrotic cell events, however, another fluorochrome is used [propidium iodide (PI)]. If a cell is dead, then PI can enter through the cell membrane of a cell, and actively bind to its nucleic acids, whereas alive cells will not allow uptake of PI. If cells are in early apoptosis, they will be FITC single positive events; if cells are in late apoptosis,

they will be FITC/PI double positive events; lastly, if cells are necrotic, they will be PI single positive events when analyzed by flow cytometry. In order to analyze events, cells were incubated for 15 minutes (protected from light) with the addition of 5μL of reagents. These samples were eventually brought up to a volume of 400μL using binding buffer. All samples were analyzed with a BD FACSCalibur flow cytometer (BD Biosciences).

Flow Cytometric Immunophenotyping

Splenocytes ($1x10^6$), obtained from NOD male and female mice exposed for an acute or chronic period of time to PCB-153, were isolated and analyzed by use of flow cytometry via methods previously described (Cetkovic-Cvrlje *et al.*, 2002; Cetkovic-Cvrlje *et al.*, 2004). The flow cytometer contains six different channels, which can be used to discriminate cells from one another. The first and second channels are side scatter (SSC) and forward scatter (FSC), which sorts cells based on size and granularity. These two channels are fluorochrome- independent. The other four channels: FL1, FL2, FL3, and FL4 require a fluorochrome conjugated with an antibody to distinguish cell populations. The fluorochromes by which the antibodies were conjugated, included: Fluoroscein isothiocyanate (FITC), Phycoerythrin (PE), Peridinin chlorophyll (PerCP), and Allophycocyanin (APC). FL1 is associated with FITC, FL2 is associated with PE, FL3 is associated with PerCP, and FL4 is associated with APC.

Splenocytes were labeled by antibodies (bound to fluorochromes), specifically directed towards certain cell markers. These fluorochromes were

readily excited by a laser, releasing a photon of light, which was then be detected by the secondary detection system. This type of detection allowed identification and quantification of cell types by cell markers. The cell markers used in this analysis included: CD3 (total T-cells), CD4 (T-helper cells), CD8 (T-cytotoxic cells), B220 (B-cells), CD11b (macrophages), NK1.1 (natural killer cells), and CD4/ CD25 (T-regulatory cells).

For detection of these markers, the following fluorochrome-labeled antibodies were used: anti-CD4 PerCP (clone RM4-5), anti-NK1.1 FITC (clone PK136), anti-CD3 PE (clone 145-2C11), anti-CD11b PerCP (clone M1/70), and anti-B220 APC (clone RA3-6B2), anti-CD8 FITC (clone 53-6.7), and anti-CD25 APC (clone 3C7) (BD Biosciences). All antibodies were diluted 1:100 in FACS buffer (0.1% NaN_3, 1% Fetal Calf Serum, and PBS), and placed in FACS polystyrene tubes with the $1x10^6$ cells. Then, the cell and antibody mixtures were incubated at 4°C for 30 minutes, protected from light. After the incubation, the cells were washed three times by intermittent decanting of supernatant, suspension in FACS buffer, and centrifugation at 1200rpm for five minutes. After the final washing, cells were suspended into 350μL of FACS buffer, and analyzed by a BD FACSCalibur flow cytometer and CellQuest Pro analyzing software.

Cytokine Analysis

Single cell suspensions were prepared from the spleens of NOD mice exposed to PCB-153 during the acute or chronic experimental period were performed. Cells were added into appropriate wells of a 24-well plate in the amount

of 500μL (to achieve a final concentration of $4x10^6$ cells/mL), and were either unstimulated (mixed with another 500μL complete media) or stimulated (mixed with 500μL of 3μg/mL ConA solution). These cells were cultured for 48 hours in a growth chamber at 37°C and 5% CO_2, and then spun in a centrifuge at 1200rpm for 10 minutes. The supernatants (containing the secreted cytokines) were extracted from the wells, leaving behind the cells, and subsequently divided into 250μL aliquots. These samples, contained in microfuge tubes, were placed into a -80°C freezer until ready for analysis.

Quantification of cytokines present in each sample was performed by use of flow cytometry and a BD™ CBA Mouse $T_{H1}/T_{H2}/T_{H17}$ Cytokine Kit (purchased from BD Biosciences). Cells were analyzed according to manufacturer instructions, but general methodology will be described. First, lyophilized cytokine top standard was reconstituted with 2.0mL of manufacturer provided Assay Diluent. Then, this solution was serially diluted 1:2 until the final dilution was reached at 1:126. The capture beads (beads which are conjugated with antibodies specific for certain cytokines) were added in the amount of 10μL to one tube, which served as the total capture bead mixture. The total capture bead mixture was added to each tube of the serial dilution, in the amount of 50μL, followed by addition of 50μL thawed cell samples. Lastly, the detection agent (fluorochrome PE) was added to each assay tube in the amount of 50μL, and then the solutions were allowed to incubate for two hours. After incubation, all samples were acquired using a BD FACSCalibur flow cytometer and appropriate templates, and analyzed using FCAP Array

software (SoftFlow, New Brighton, MN, USA). From this, the presence of cytokines: IL-2, -4, -6, -10, -17, IFN-γ, and TNF-α in each sample was able to be determined.

Statistical Analysis

Differences in diabetes incidence amongst experimental groups were determined by use of life-table analyses and log-rank test (Mantel-Cox), using the statistical software program SPSS (IBM, Armonk, NY, USA); p-values<0.05 were considered statistically significant. For blood glucose levels, and body weights, a one-way ANOVA with repeated measures was used, with a p-value<0.05 being statistically significant. A one-way ANOVA was performed on all PCB-153 drug dose dependency *in vitro* experiments, to determine differences between the different concentrations. If the p-value was less than 0.05, Tukey's post-hoc tests were performed, with a p-value<0.05 being statistically significant. For all other experiments (immunophenotyping, T-cell proliferation, apoptosis detection) student's t-tests were performed, with a p-value<0.05 being statistically significant.

Chapter V

RESULTS

In Vitro Effects of PCB-153 on T-Cell Function

Given the clear association between ortho-substituted PCBs and thymocyte atrophy (Tan et al., 2003), speculations about PCB-153 (a di-ortho-substituted PCB) and its effects on T-cell function were raised. To identify the relationship between the PCB- 153 and T-cell functionality, an assay was performed using splenocytes from untreated NOD mice. A serial dilution of PCB-153 was prepared in complete media, and when added to the 96-well plate along with murine splenocytes and a T-cell mitogen concanavalin A (ConA), resulted in final concentrations ranging from 100μg/mL to 0.4μg/mL. It was shown that PCB-153 significantly reduces proliferation of T-cells, with the effective doses of 100μg/mL and 50μg/mL ($p=7.64x10^{-5}$ and $p=1.84x10^{-4}$, respectively), with functional recovery achieved from 25μg down to 0.4μg (Figure 3A). The positive control received DMSO (the diluent for stock PCB-153) in the same concentration as the highest PCB-153 culture (100μg/mL), and no differences in proliferation were observed, confirming that DMSO has no effect on T-cells at that concentration.

Studies have shown that PCBs are able to evoke apoptosis in several organisms in an organ-specific manner (Hwang *et al.*, 2001; Sanchez-Alonso *et al.*, 2003; Shin *et al.*, 2000). In order to determine whether the reduced proliferation of T-cells was caused by apoptotic induction, the Annexin-V flow cytometric-based assay was performed after the 24-hour incubation with addition of 50, 12.5, and 1.56μg/mL PCB-153. These PCB-153 concentrations were chosen as a representative concentrations that were shown to be able to reduce (50μg/mL) or not reduce (12.5μg/mL and 1.56μg/mL) T-cell proliferation (Figure 3A). Annexin-V/PI staining revealed a significant difference between the 50μg/mL dose and all other PCB-153 doses and controls tested (Figure 3B). Alive cells (PI^-Annexin-V-$FITC^-$) were significantly reduced in the cultures exposed to 50μg/mL PCB-153 compared to 12.5μg/mL PCB-153, 1.56μg/mL PCB-153, positive (containing DMSO) and negative control (no DMSO addition) ($p=1x10^{-4}$, $2x10^{-4}$, $p=4.5x10^{-4}$, and $4.1x10^{-4}$, respectively), whereas apoptotic (PI^+Annexin-V- $FITC^+$) events were significantly increased in the same cultures exposed to 50μg/mL PCB-153 compared to all other doses of PCB-153 exposure, positive, and negative control ($p=0.016$, 0.0067, $p=0.0066$, and $2.3x10^{-4}$, respectively) (Figure 3B).

These data suggest that PCB-153 is able to inhibit T-cell proliferation *in vitro* at higher doses through induction of apoptosis.

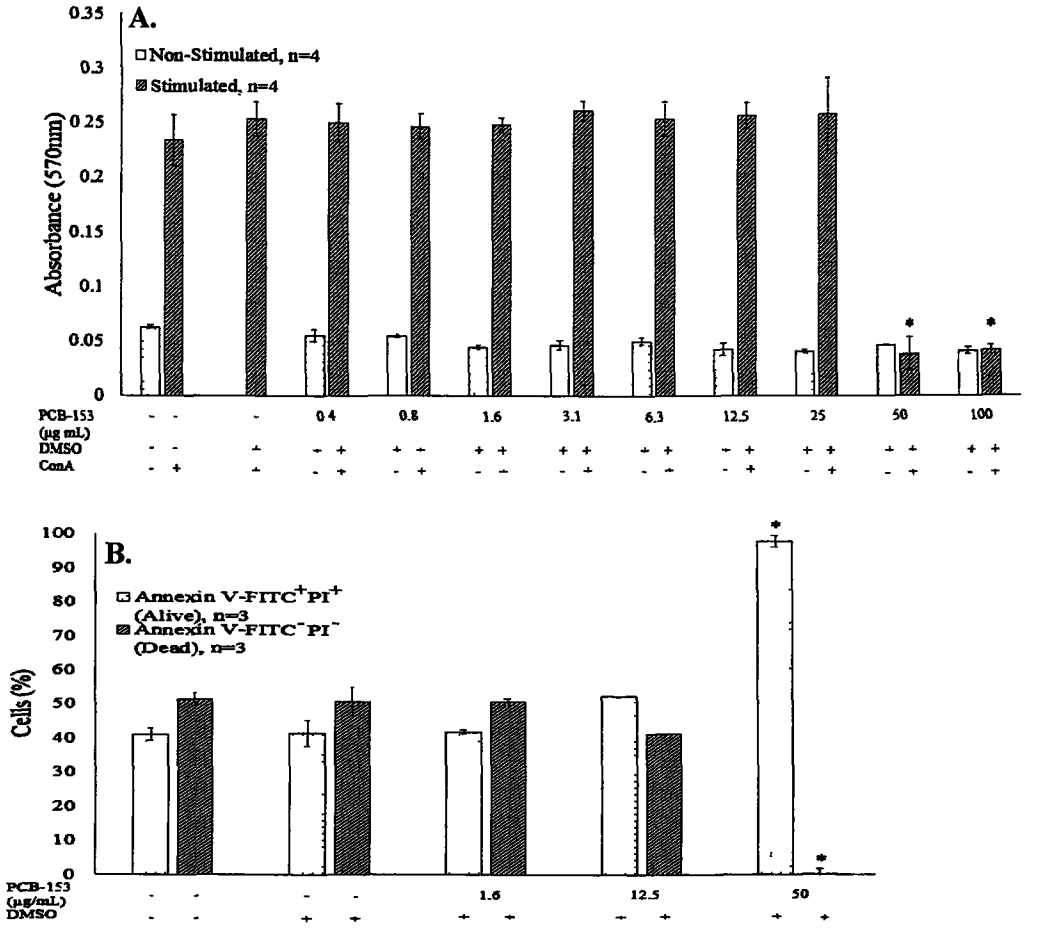

Figure 3. Proliferation of T-cells (**A**) and detection of apoptosis (**B**) after PCB-153 exposure, *in vitro*. **(A)** Cultures of NOD splenocytes were exposed to PCB-153 in the absence (non-stimulated) or presence of ConA (3µg/mL) (stimulated). After 72 hours of incubation, Alamar Blue reagent was added to cultures and absorbance measured at 570nm. Concentrations of PCB-153 ranged from 100µg/mL to 0.4µg/mL; positive control contained DMSO in the same concentration present in the highest PCB-153 concentration culture (100µg/mL); the negative control consisted solely of culture medium. Data are presented as mean ± SEM, with significance determined by a one-way ANOVA and Tukey's post-hoc test (*$p<0.05$). **(B)** Detection of apoptosis in NOD splenocytes cultured for 24 hours with PCB-153 (100-1.56µg/mL) was performed by the Annexin-V FITC assay; positive controls received DMSO in the same concentration as the highest PCB-153 culture; negative controls were exposed to medium. 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 presented as mean ± SEM, with significance determined by student's T-test (*$p<0.05$).

Effects of Acute Exposure to PCB-153 on Immune Parameters of NOD Mice

Given that the data obtained in the *in vitro* experiments showed an ability of PCB-153 to reduce proliferation of T-cells, it became feasible to test the effects of PCB-153 on mice with acute exposure to PCB-153, in an effort to conceive the immediate immune-related effects of the compound. Eight-week-old males were used for the acute exposure since they do not develop diabetes as early as females, thereby circumventing any potential effects disease occurrence may have on the immune parameters chosen for study. The aim of the acute exposure was to determine whether PCB-153, at high (50mg/Kg)- and low (0.5mg/Kg)-doses, acts on T-cells *in vivo*. Splenocytes of mice exposed to PCB-153 were tested for: total cell counts, viability, immunophenotyping of major T-cell subsets, as well as other immune cell types, proliferation, and cytokine profiles.

Total cell counts ($x10^6$) were significantly reduced in the high-dose PCB-153 exposed group ($p=0.04$), compared to controls (Figure 4A); whereas viability of splenic cells obtained from all groups tested were not significantly different from one another (Figure 4B). Immunophenotyping by use of flow cytometry, in terms of percentages, showed a significant decrease in $CD4^+$ T-cells in the low-dose group ($p=0.005$), and a decreased trend in $CD4^+$ T-cells in the high-dose group, compared to controls; a significant increase in macrophages was observed in the high-dose group compared to controls ($p=0.008$), too (Figure 5A). Immunophenotyping by total cell counts revealed significantly reduced $CD4^+$ T-cells in the 50mg/Kg treatment group compared to controls ($p=0.041$). Also in

the 50mg/Kg group, a significant decrease in the population of $B220^+$ B-cells was observed, compared to controls (p=0.024) (Figure 5B). Analysis of ConA-induced T-cell proliferation revealed a significant reduction in the 50mg/Kg PCB-153-exposed group compared to controls ($p=1.19x10^{-6}$) (Figure 6).

Evaluation of the cytokines, obtained from splenocytes of the PCB-153-exposed mice, showed significant decreases in all cytokines tested in the low-dose group (IL-10: p=0.009; IL-17A: p=0.05; TNF-α: p=0.009; IFN-γ: p=0.038; IL-6: p=0.007; IL-4: p=0.0006; IL-2: p=0.018). Interestingly, only IL-2 was significantly reduced in the high-dose group (p=0.04), with a trend of lowering levels of TNF-α and IL-6 without reaching significance (Figure 7).

Overall, these data suggest a prominent *in vivo* immunosuppressive property of high-dose PCB-153-exposure, defined by significantly reduced splenocytes' counts, absolute numbers of $CD4^+$ T-cells, T-cell proliferation ability and IL-2 secretion. Interestingly, whereas low-dose PCB-153 treatment did not affect splenocyte counts, absolute $CD4^+$T-cell numbers and T-cell proliferation, it *did* significantly reduce $CD4^+$ T-cell percentages, as well as the levels of all cytokines studied.

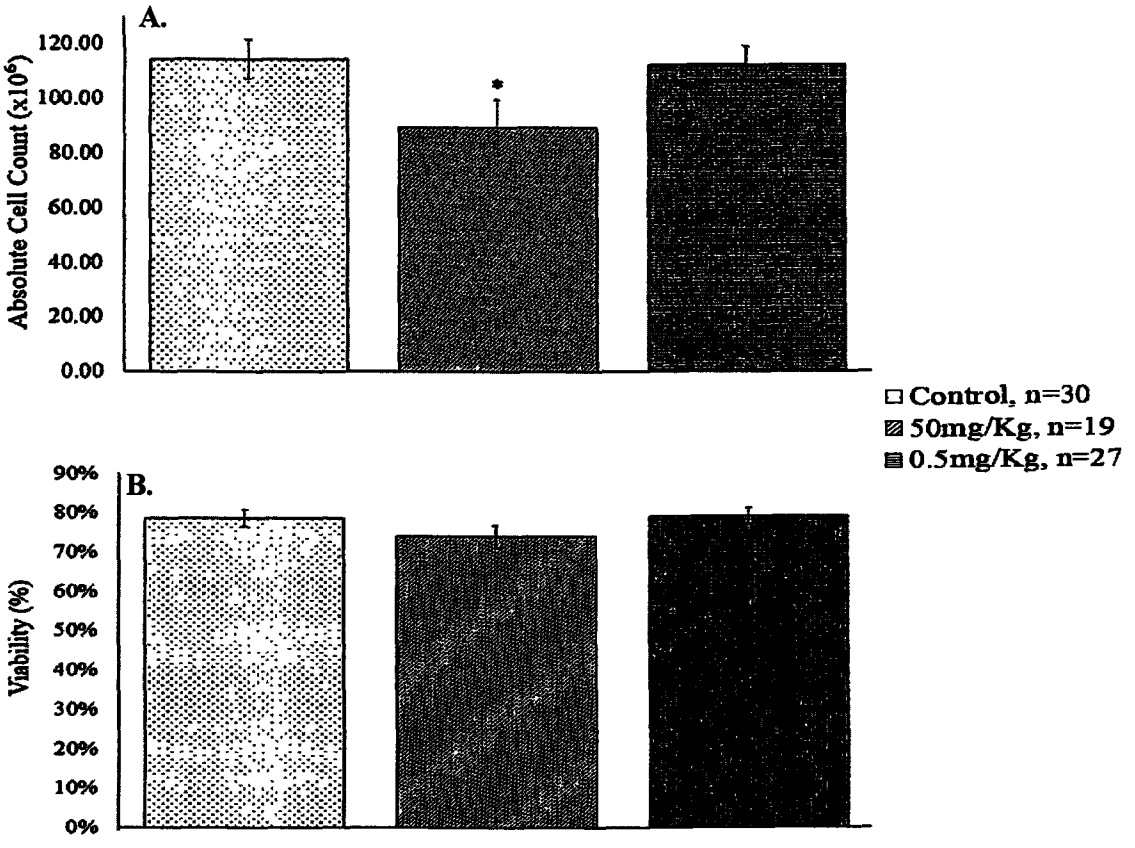

Figure 4. Total splenic cell counts (**A**) and viability (**B**) after acute exposure of NOD males to PCB-153. Male NOD mice were treated with PCB-153 (0.5 or 50mg/Kg) or corn oil vehicle control, for five injections on day 1, 3, 5, 7, and 9, and sacrificed on day 10. **(A)** Total cell counts ($x10^6$) were obtained by use of the Trypan Blue exclusion method. **(B)** Cell viability was determined by use of Trypan Blue exclusion method and average viability percentages were calculated (based on alive cells/total cells). Data are presented as mean ± SEM with significance determined by student's T-test (*$p<0.05$).

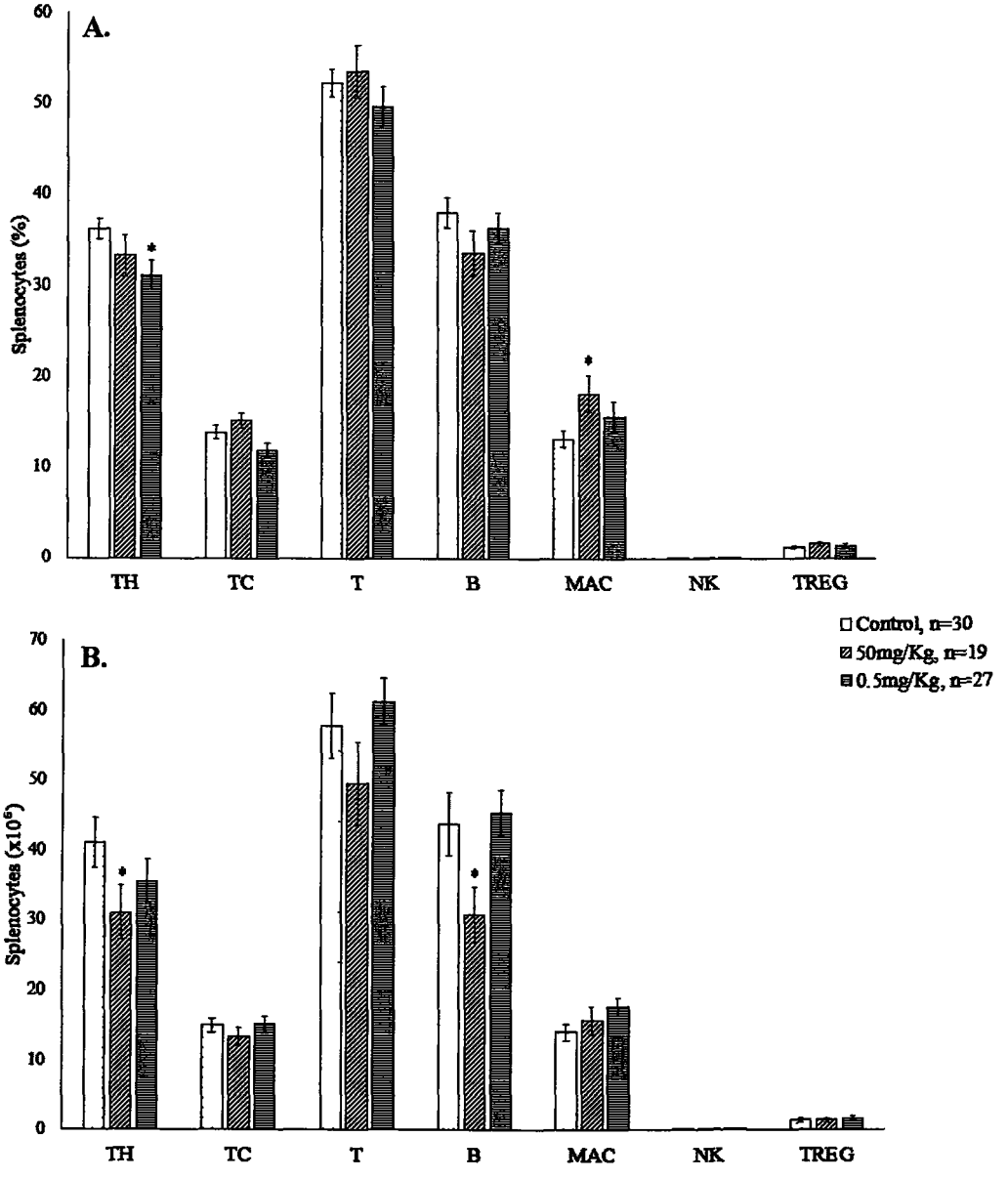

Figure 5. Immunophenotyping of NOD splenocytes after acute exposure to PCB-153. **(A)** Percentages; and **(B)** Total numbers ($x10^6$) of splenic CD4 (TH), CD8 (TC), CD3 (total T-cells; T), CD45RB220 (B-cells; B), CD11b (MAC), NK1.1 (NK), and CD4+CD25+ (TREG) cells. Male NOD mice were treated with PCB-153 as outlined in the legend of Figure 4. Splenocytes were stained with antibodies against various cell markers and analyzed by flow cytometry. Data are presented as mean ± SEM with significance determined by student's T-test (*$p<0.05$).

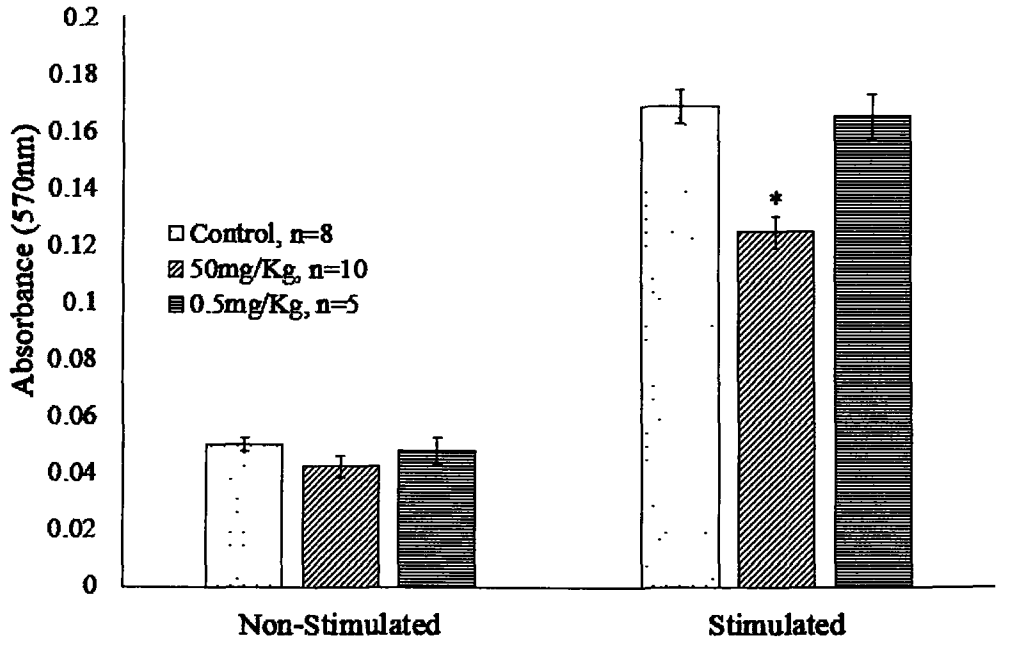

Figure 6. Proliferation of T-cells obtained from male NOD mice after acute exposure to PCB-153. Male NOD mice were treated as outlined in Figure 4. Splenocytes were cultured in the absence (non-stimulated) or presence (stimulated) of ConA (3μg/mL) for 72 hours. Proliferation of T-cells was measured by use of the Alamar Blue colorimetric assay, and absorbance determined at 570 nm. Data are presented as mean ± SEM with significance determined by student's T-test (*$p<0.05$).

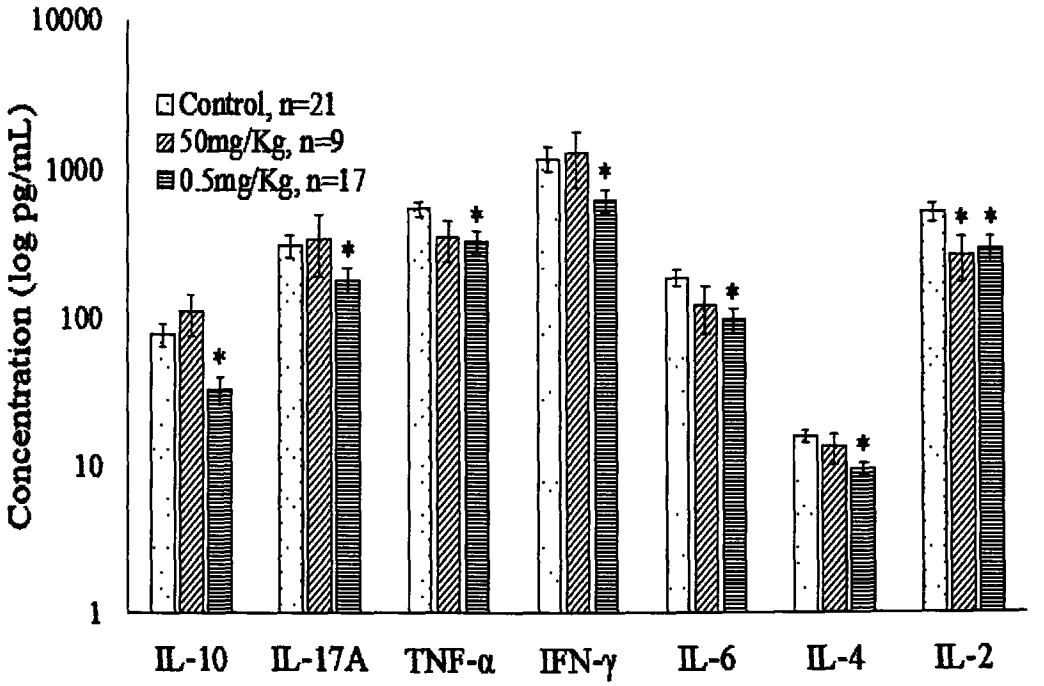

Figure 7: Quantification of cytokine levels after acute exposure of NOD males to PCB-153. Cytokines acquired from supernatants of splenocytes obtained from mice exposed to 0.5 or 50 PCB-153 mg/Kg as outlined in the legends to Figure 4. After 48 h of culture in the presence of ConA (3μg/mL), supernatants were analyzed using a CBA mouse $T_{H1}/T_{H2}/T_{H17}$ kit that permits quantification of IL-6, TNF-α, IFN-γ, IL-2, IL-4, IL-10 and IL-17A. Data shown are mean ± SEM; with significance determined by student's T-test (*$p<0.05$).

Effects of Chronic PCB-153 Exposure on T1D Incidence and Glycemia Levels in NOD Mice

Given the observed immunosuppressive effects of *in vivo* acute PCB-153 exposure, it was decided to test whether chronic exposure to PCB-153 would affect development of T1D in NOD females. Non-diabetic, healthy, eight-to-nine (8-9)-week old NOD females were treated intraperitoneally with either: 12.5mg/Kg PCB-153 (high-dose, n=24), 0.125mg/Kg PCB-153 (low-dose, n=23), or control (n=25)

injections twice per week, until they became diabetic or to the 16-week endpoint (24-weeks of age), whichever came first. All experimental mice were checked to confirm euglycemia at the start of the experiment, so that no artificial bias was evoked when randomly assigning mice to treatment groups. During the course of the 16-week treatment period, no signs of toxicity (decrease in body weights or clinical signs and symptoms of ongoing pathologic process) were observed in any of the aforementioned experimental groups. However, two mice from the control group were removed from the experiment; one mouse died for unknown reasons during the second week of treatment, while the other had developed a large debilitating tumor by the thirteenth-week of treatment. Blood glucose measurements were taken weekly from all experimental mice up to the 16-week endpoint. The overall incidence of T1D was significantly decreased in both the high- and low-dose PCB-153 exposed groups, with the high-dose being the most significant ($p=0.002$ and $p=0.022$, respectively), compared to controls (Figure 8A). At 9-weeks of treatment, 50% of control mice were diabetes free, whereas the low-dose group reached similar percentages at fifteen weeks of treatment. By the 16-week endpoint, the control group was only 28% disease free, whereas the 12.5mg/Kg and 0.125mg/Kg groups were 66.7% and 48% disease free, respectively. The high-dose PCB-153-exposed group showed a trend of lower glycemia levels compared to the controls, however statistical significance was not reached ($p=0.08$) (Figure 8B). Body weights of NOD females treated chronically for up to 16-weeks with PCB-153 were increased by the endpoint compared to

controls, with the most apparent increase being observed in the high-dose group (though not reaching statistical significance) (Figure 8C). From this, indication towards protection from T1D onset in PCB-153 treated mice is apparent, with the high-dose PCB-153-treated group (12.5mg/Kg) exhibiting the most prominent protective effects.

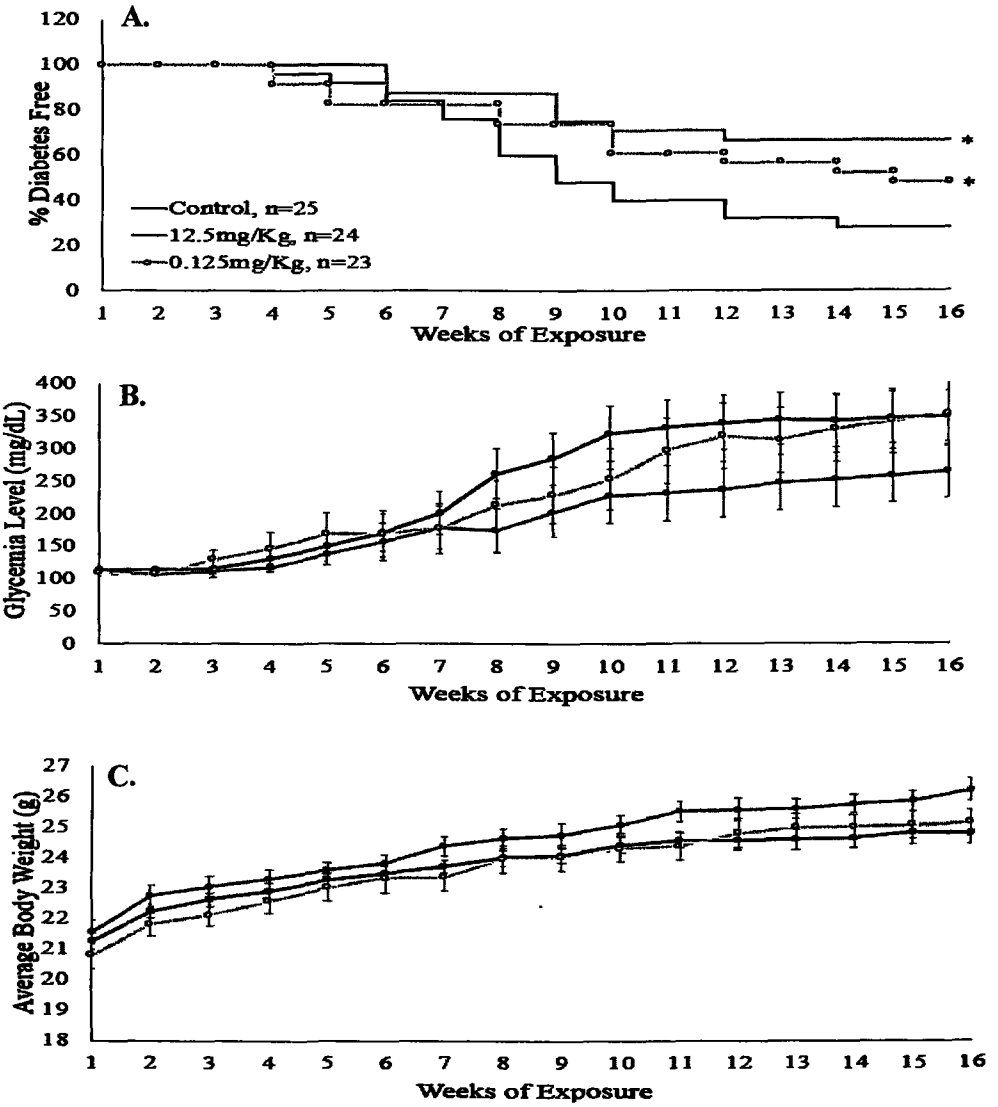

Figure 8. Diabetes incidence (**A**), average glycemia levels (**B**), and body weights (**C**) in NOD females exposed to PCB-153 up to 24-weeks-of-age (16 weeks of exposure). Mice received PCB-153 at 0.125 or 12.5 mg/Kg intraperitoneal (i.p.) injections biweekly, starting at 8 weeks-of-age; control mice received vehicle. (**A**) Diabetes incidence presented as fractional disease incidence, where 1.0 represents 100% diabetes-free; significance determined through Mantel-Cox log-rank test (*$p<0.05$). (**B**) Glycemia determined on a weekly basis. Blood glucose measurements were recorded weekly with two consecutive measurements of 220mg/dL confirming onset of diabetes. Data shown are mean ± SEM. (**C**) Average body weight (g) of NOD females treated up to 16 weeks with PCB-153 were recorded weekly; data shown are mean ± SEM.

Effects of Chronic PCB-153 Exposure on the Immune Parameters of NOD Mice During Disease Development

Based upon the ability of PCB-153 to decrease the incidence of autoimmune T1D in chronically-exposed NOD females, and induce immunosuppressive effects in acutely-exposed NOD males, it was next asked what the underlying mechanism of PCB-153 action during the development of disease may be. Eight-week-old prediabetic NOD females were treated using the same experimental design, doses, and regimen of PCB-153 administration described in the experiments that followed the incidence of diabetes (12.5mg/Kg PCB-153, 0.125mg/Kg PCB-153, or vehicle control injected twice a week), but were sacrificed at three different endpoints (2, 8, and 16-weeks) during the disease development.

NOD females treated up to 2-weeks with PCB-153, in either dose, showed no significant differences in cell viability (Figure 9A); however a trend in increase of total splenic cell counts ($x10^6$) was observed in both PCB-153-exposed animals, with a significance reached in the high-dose group, compared to the control ($p=0.026$) (Figure 9B). Immunophenotyping revealed a significant decrease in percentages of $CD4^+$ cells in both the high- and low-dose groups compared to controls ($p=0.006$ and 0.04, respectively) (Figure 10A). However, a reduction of the absolute numbers of $CD4^+$ T-cells was not observed when data were calculated based on total cells ($x10^6$) (Figure 10B). The proliferative response of T-cells from the low-dose group to mitogen (ConA) was significantly reduced, compared to

controls, (p=0.003), with no difference observed in the high-dose group (Figure 11). Cytokine analysis showed a significant decrease in TNF-α in the high-dose group (p=0.05) and a trend of decrease in the low-dose group, while the low-dose group had a significant decrease in IL-6 (p=0.028), with a trend towards decrease observed in the high-dose group (Figure 12). There were no differences observed in any other cytokine analyzed.

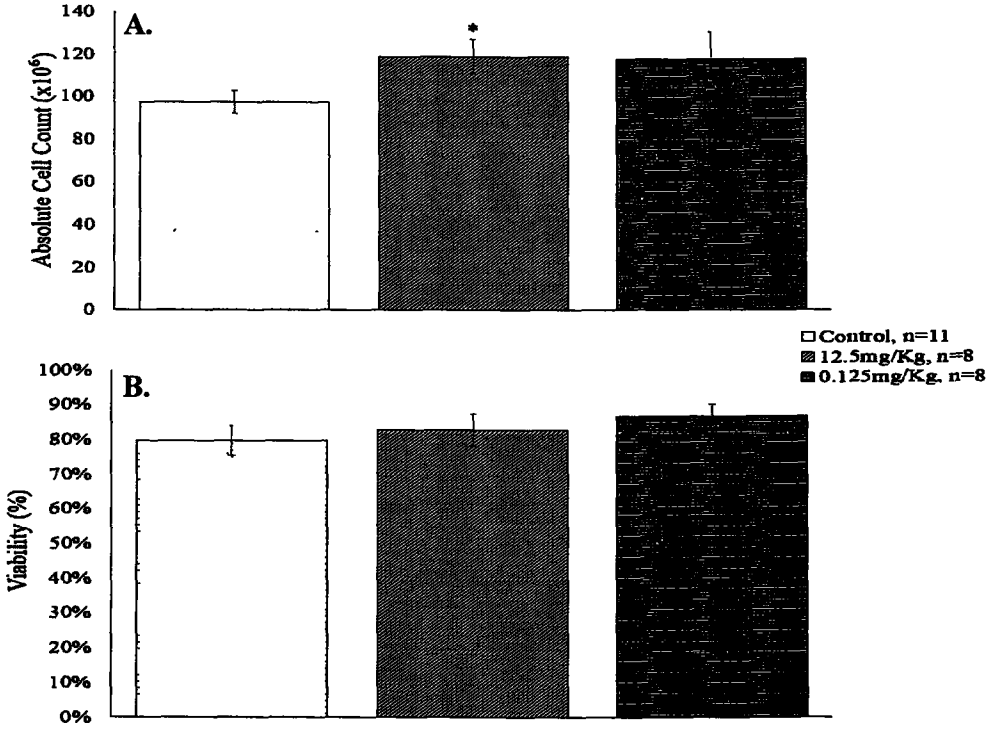

Figure 9. Total splenic cell counts (**A**) and viability (**B**) after chronic exposure of NOD females to PCB-153, up to 2-weeks. Female NOD mice were treated with PCB-153 (0.125 or 12.5mg/Kg) or corn oil vehicle control, for up to 2-weeks, with injections occurring i.p., biweekly. **(A)** Total cell counts ($x10^6$) were obtained by use of the Trypan Blue exclusion method. **(B)** Cell viability was determined by use of Trypan Blue exclusion method and average viability percentages were calculated (based on alive cells/total cells). Data are presented as mean ± SEM with significance determined by student's T-test (*$p<0.05$).

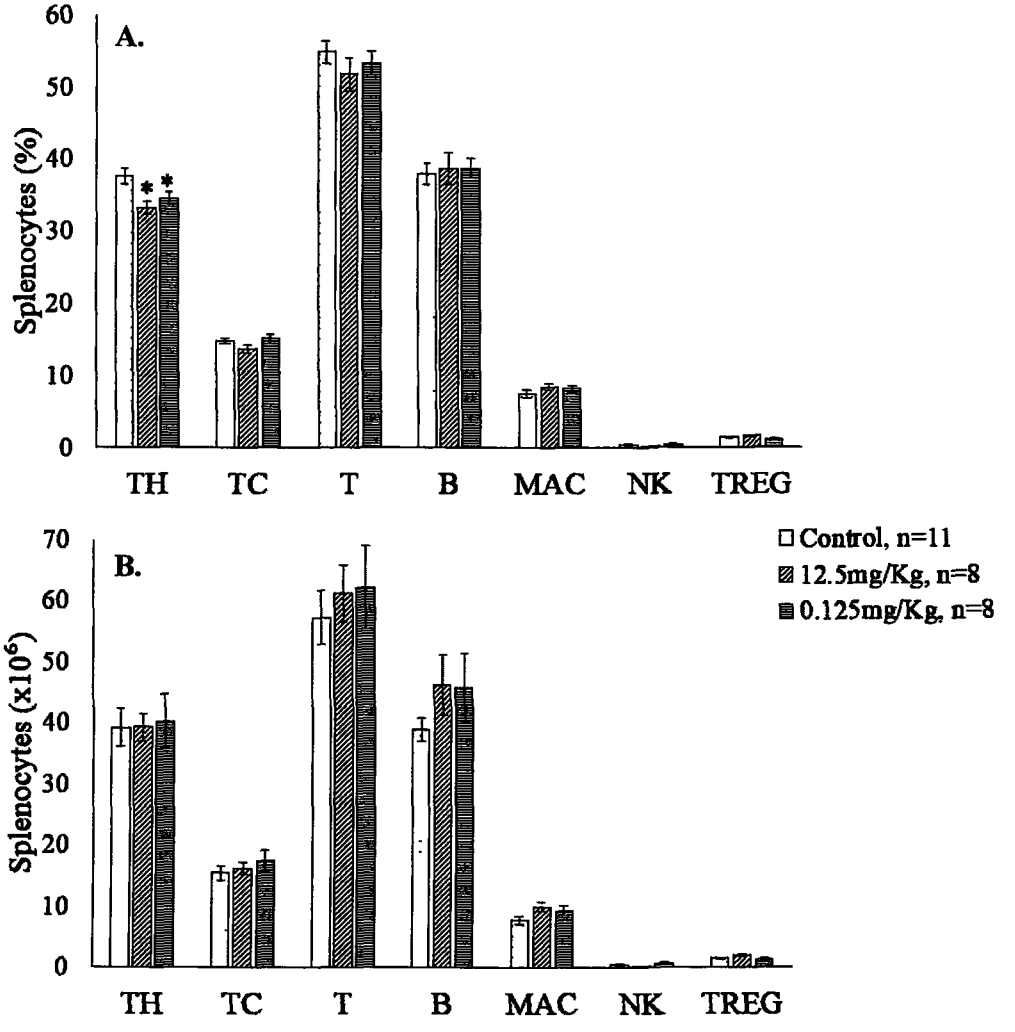

Figure 10. Immunophenotyping of NOD splenocytes after chronic exposure to PCB-153, up to 2-weeks. **(A)** Percentages; and **(B)** Total numbers ($x10^6$) of splenic CD4 (TH), CD8 (TC), CD3 (total T-cells; T), CD45RB220 (B-cells; B), CD11b (MAC), NK1.1 (NK), and CD4+CD25+ (TREG) cells. Female NOD mice were treated with PCB-153 as outlined in the legend of Figure 9. Splenocytes were stained with antibodies against various cell markers and analyzed by flow cytometry. Data are presented as mean ± SEM with significance determined by student's T-test (*$p<0.05$).

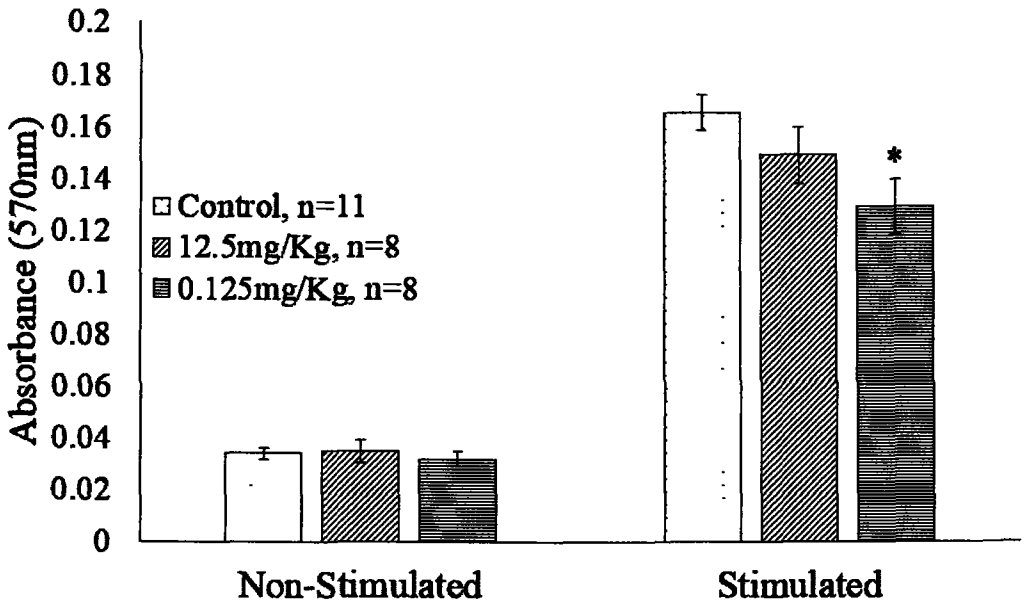

Figure 11. Proliferation of T-cells obtained from female NOD mice after chronic exposure to PCB-153, up to 2-weeks. Female NOD mice were treated as outlined in Figure 9. Splenocytes were cultured in the absence (non-stimulated) or presence (stimulated) of ConA (3μg/mL) for 72 hours. Proliferation of T-cells was measured by use of the Alamar Blue colorimetric assay, and absorbance determined at 570 nm. Data are presented as mean ± SEM with significance determined by student's T-test (*$p<0.05$).

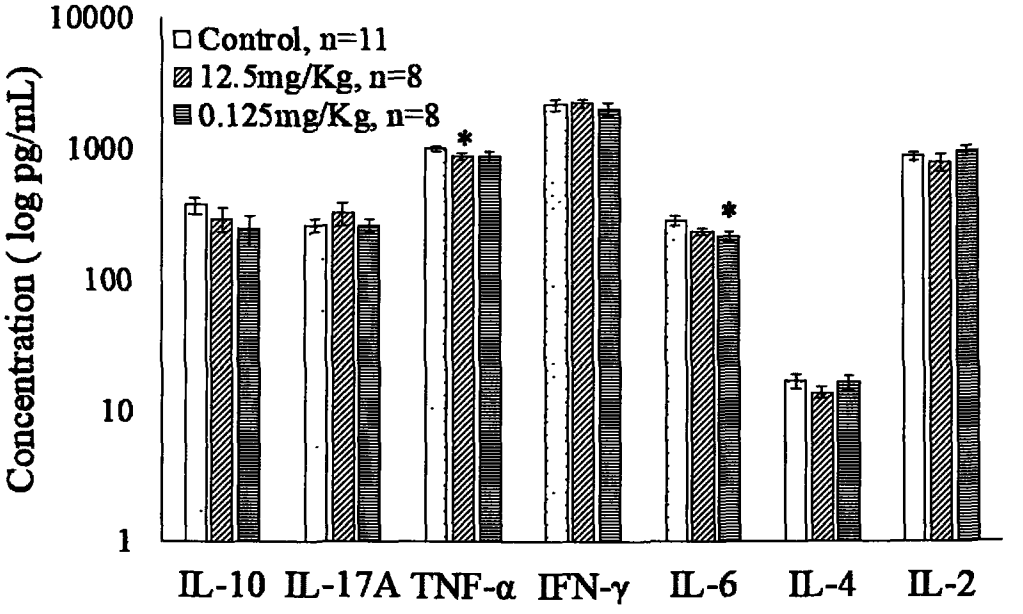

Figure 12. Quantification of cytokine levels after chronic exposure of NOD females to PCB-153, up to 2-weeks. Cytokines acquired from supernatants of splenocytes obtained from mice exposed to 0.125 or 12.5 PCB-153 mg/Kg as outlined in the legend of Figure 9. After 48 h of culture in the presence of ConA (3μg/mL), supernatants were analyzed using a CBA mouse $T_{H1}/T_{H2}/T_{H17}$ kit that permits quantification of IL-6, TNF-α, IFN-γ, IL-2, IL-4, IL-10 and IL-17A. Data shown are mean ± SEM; with significance determined by student's T-test (*$p<0.05$).

At the 8-week endpoint, no differences in total cell counts ($x10^6$) and cell viabilities were observed in either PCB-153-exposed group, except for a slight decrease in cell viability of cells from the low-dose group ($p=0.044$) (Figures 13A and B). Immunophenotyping of the splenic cell samples, in terms of percentages, showed significant decreases in $CD4^+$ and $CD3^+$ T-cells in the low-dose group, compared to controls ($p=9.75x10^{-5}$ and $p=0.03$, respectively). No significant differences were observed in the high-dose group (Figure 14A). Similarly, in terms of total cell counts, a significant decrease in the $CD4^+$ cell subset ($p=0.03$) along

with a decrease in the $CD3^+$ cell subset (though statistical significance was not achieved, p=0.07), was observed in the same PCB-153-exposed group (Figure 14B). A similar trending towards decrease in the percentages and absolute numbers of the $CD4^+$ cell subset in the high-dose group was observed, though statistical significance was not achieved (Figure 14A and B). T-cell proliferation revealed a significant reduction in the low-dose group, compared to controls (p=0.03), with no differences observed in the high-dose group (Figure 15). Decreases in TNF-α and IL-2 secretions were observed in the low-dose group after cytokine analysis (p=0.011 and p=0.013, respectively), with no significance shown in any other cytokine analyzed, nor any differences in the high-dose group, compared to controls (Figure 16).

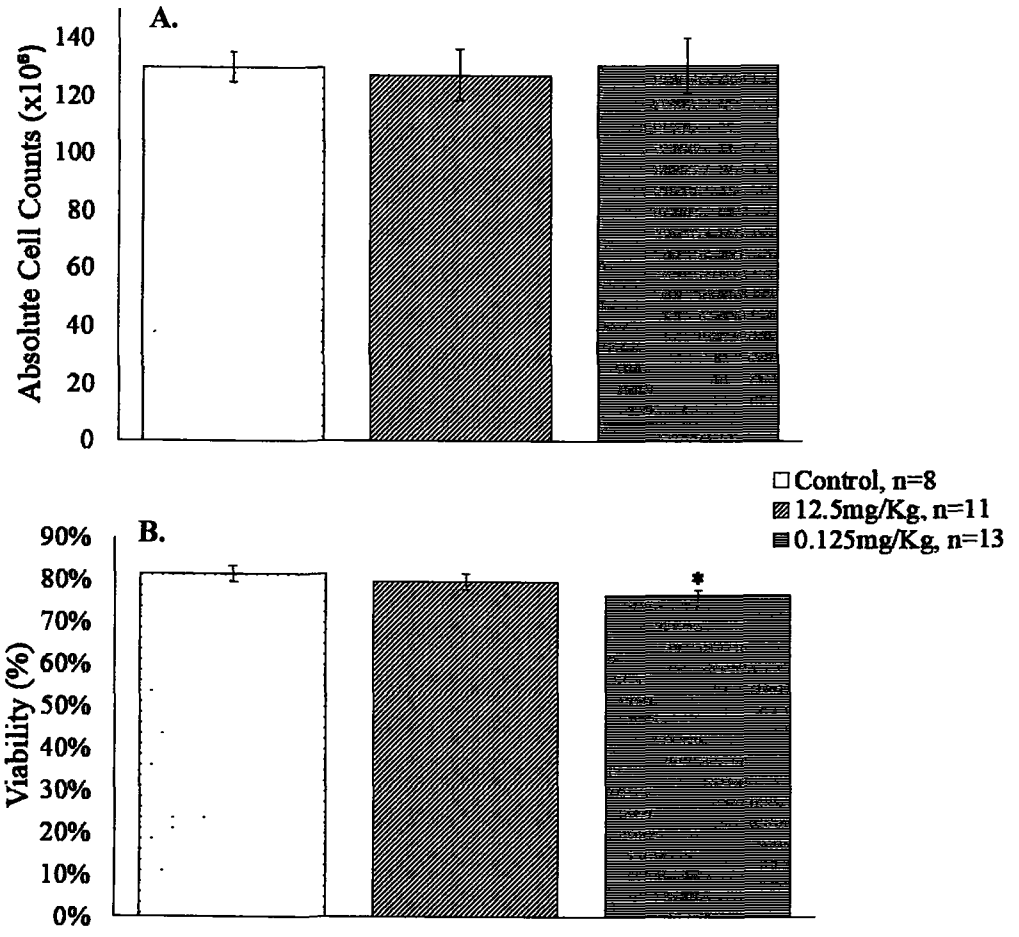

Figure 13. Total splenic cell counts (**A**) and viability (**B**) after chronic exposure of NOD females to PCB-153, up to 8-weeks. Female NOD mice were treated with PCB-153 (0.125 or 12.5mg/Kg) or corn oil vehicle control, for up to 8-weeks, with injections occurring i.p., biweekly. **(A)** Total cell counts ($x10^6$) were obtained by use of the Trypan Blue exclusion method. **(B)** Cell viability was determined by use of Trypan Blue exclusion method and average viability percentages were calculated (based on alive cells/total cells). Data are presented as mean ± SEM with significance determined by student's T-test (*$p<0.05$).

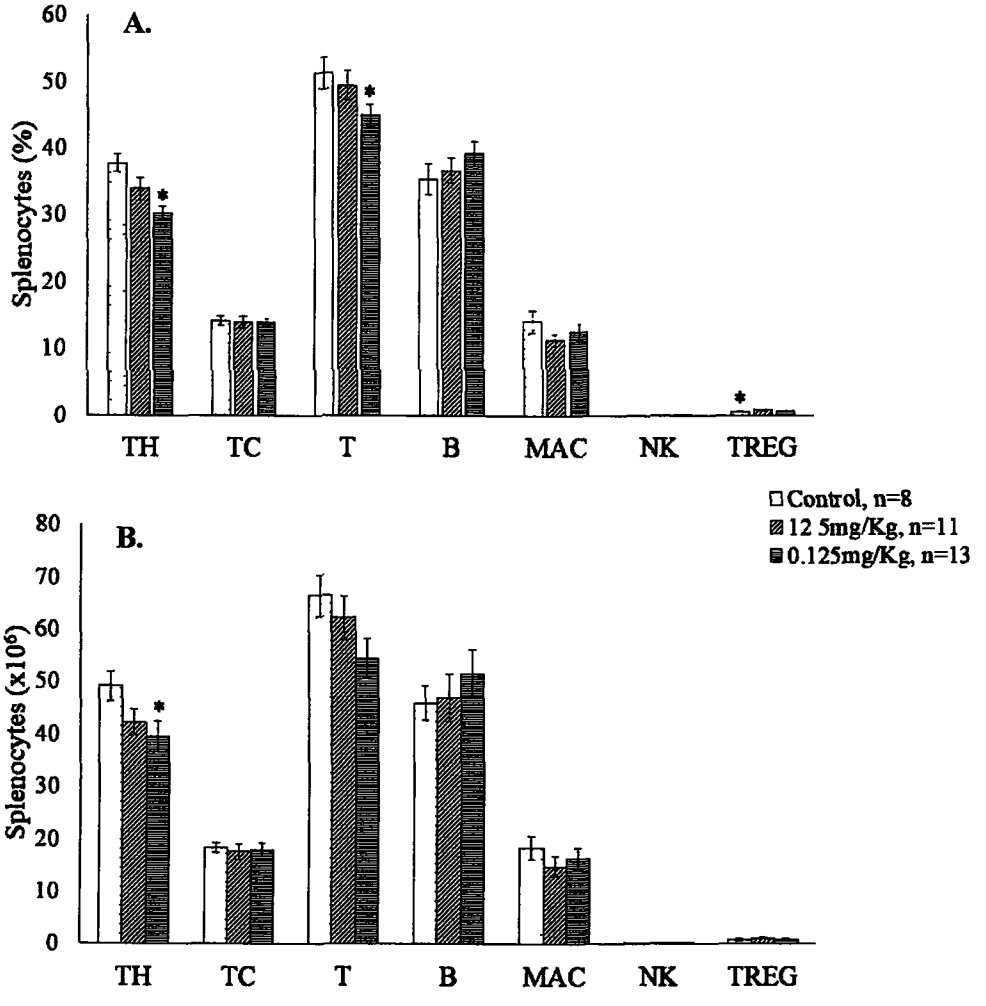

Figure 14. Immunophenotyping of NOD splenocytes after chronic exposure to PCB-153, up to 8-weeks. **(A)** Percentages; and **(B)** Total numbers (x10^6) of splenic CD4 (TH), CD8 (TC), CD3 (total T-cells; T), CD45RB220 (B-cells; B), CD11b (MAC), NK1.1 (NK), and CD4+CD25+ (TREG) cells. Female NOD mice were treated with PCB-153 as outlined in the legend of Figure 13. Splenocytes were stained with antibodies against various cell markers and analyzed by flow cytometry. Data are presented as mean ± SEM with significance determined by student's T-test (*$p<0.05$).

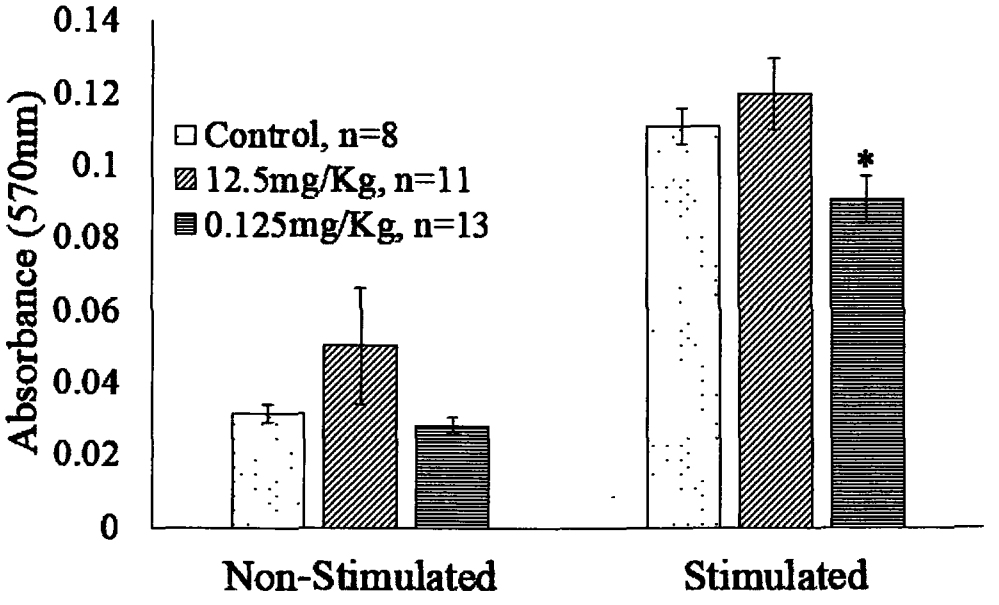

Figure 15. Proliferation of T-cells obtained from female NOD mice after chronic exposure to PCB-153, up to 8-weeks. Female NOD mice were treated as outlined in Figure 13. Splenocytes were cultured in the absence (non-stimulated) or presence (stimulated) of ConA (3μg/mL) for 72 hours. Proliferation of T-cells was measured by use of the Alamar Blue colorimetric assay, and absorbance determined at 570 nm. Data are presented as mean ± SEM with significance determined by student's T-test (*$p<0.05$).

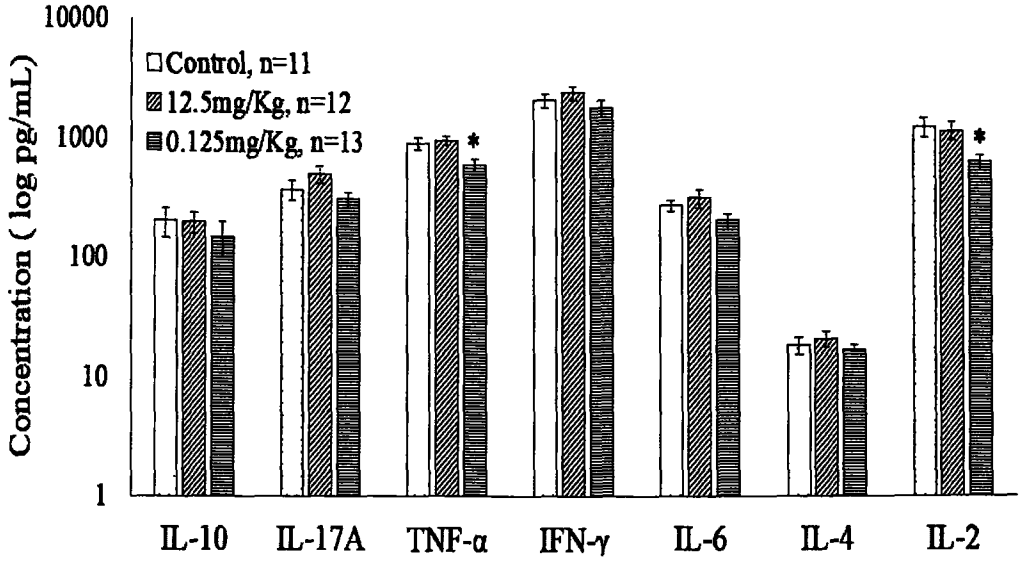

Figure 16. Quantification of cytokine levels after chronic exposure of NOD females to PCB-153, up to 8-weeks. Cytokines acquired from supernatants of splenocytes obtained from mice exposed to 0.125 or 12.5 PCB-153 mg/Kg as outlined in the legend of Figure 13. After 48 h of culture in the presence of ConA (3μg/mL), supernatants were analyzed using a CBA mouse $T_{H1}/T_{H2}/T_{H17}$ kit that permits quantification of IL-6, TNF-α, IFN-γ, IL-2, IL-4, IL-10 and IL-17A. Data shown are mean ± SEM; with significance determined by student's T-test (*$p<0.05$).

NOD females treated up to 16-weeks with PCB-153 in both doses revealed significant differences in total splenic cell counts ($x10^6$) (Figure 17A). A significant reduction in splenic cell viabilities was found in both high- and low-dose PCB-153 groups, compared to controls ($p=0.018$ and 0.019, respectively) (Figure 17B). Analysis of the immunophenotyping profiles by percentages showed a significant reduction in $CD4^+$ T-cells in both high- and low-dose PCB-153-exposed groups, compared to controls ($p=0.018$ and $p=0.007$, respectively); the low-dose group also had a significant reduction in $CD3^+$ T-cells compared to controls ($p=0.036$) (Figure 18A). Immunophenotyping by use of total cell counts revealed a trend towards

decrease in $CD4^+$ and $CD3^+$ cell subsets of both PCB-153 treated groups, with significance only being achieved in the low-dose group ($p=0.005$ and 0.02, respectively) (Figure 18B). Also in the low-dose group, $CD4^+/CD25^+$ cells were significantly reduced, compared to controls ($p=0.034$). A significant decrease in T-cell proliferation was observed in the low-dose group (stimulated), compared to both the control and high-dose groups ($p=0.003$ and $p=1.24x10^{-5}$, respectively) (Figure 19). Interestingly, the T-cells obtained from high-dose-exposed mice showed significantly increased proliferation, when both stimulated and non-stimulated groups were compared to controls ($p=0.001$ and $p=0.025$, respectively) (Figure 19). A significant decrease in TNF-α was observed in the low-dose group ($p=0.05$) with IL-2 being significantly reduced in both the high- and low-dose groups, compared to controls ($p=0.0007$ and $p=0.021$, respectively). No differences in any other cytokines analyzed were observed in either group, compared to controls (Figure 20).

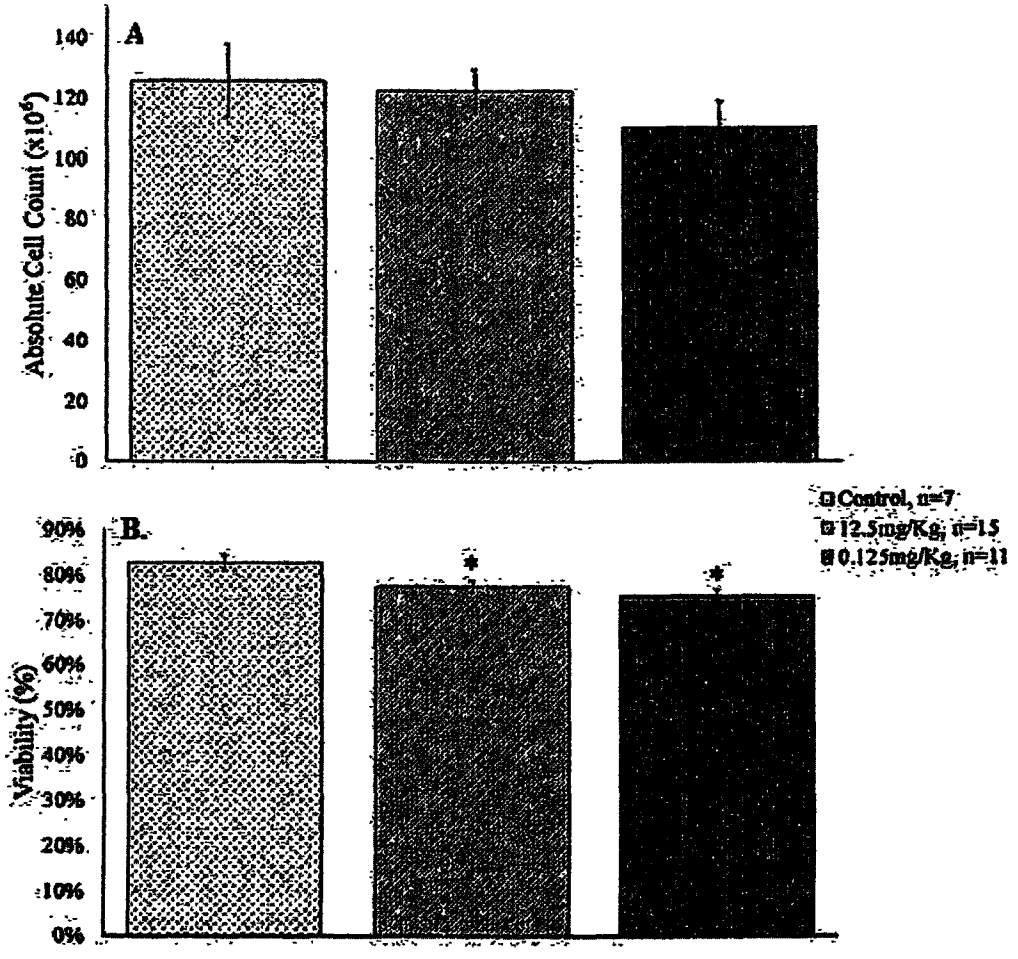

Figure 17. Total splenic cell counts (**A**) and viability (**B**) after chronic exposure of NOD females to PCB-153, up to 16-weeks. Female NOD mice were treated with PCB-153 (0.125 or 12.5mg/Kg) or corn oil vehicle control, for up to 16-weeks, with injections occurring i.p., biweekly. (**A**) Total cell counts ($x10^6$) were obtained by use of the Trypan Blue exclusion method. (**B**) Cell viability was determined by use of Trypan Blue exclusion method and average viability percentages were calculated (based on alive cells/total cells). Data are presented as mean ± SEM with significance determined by student's T-test (*$p<0.05$).

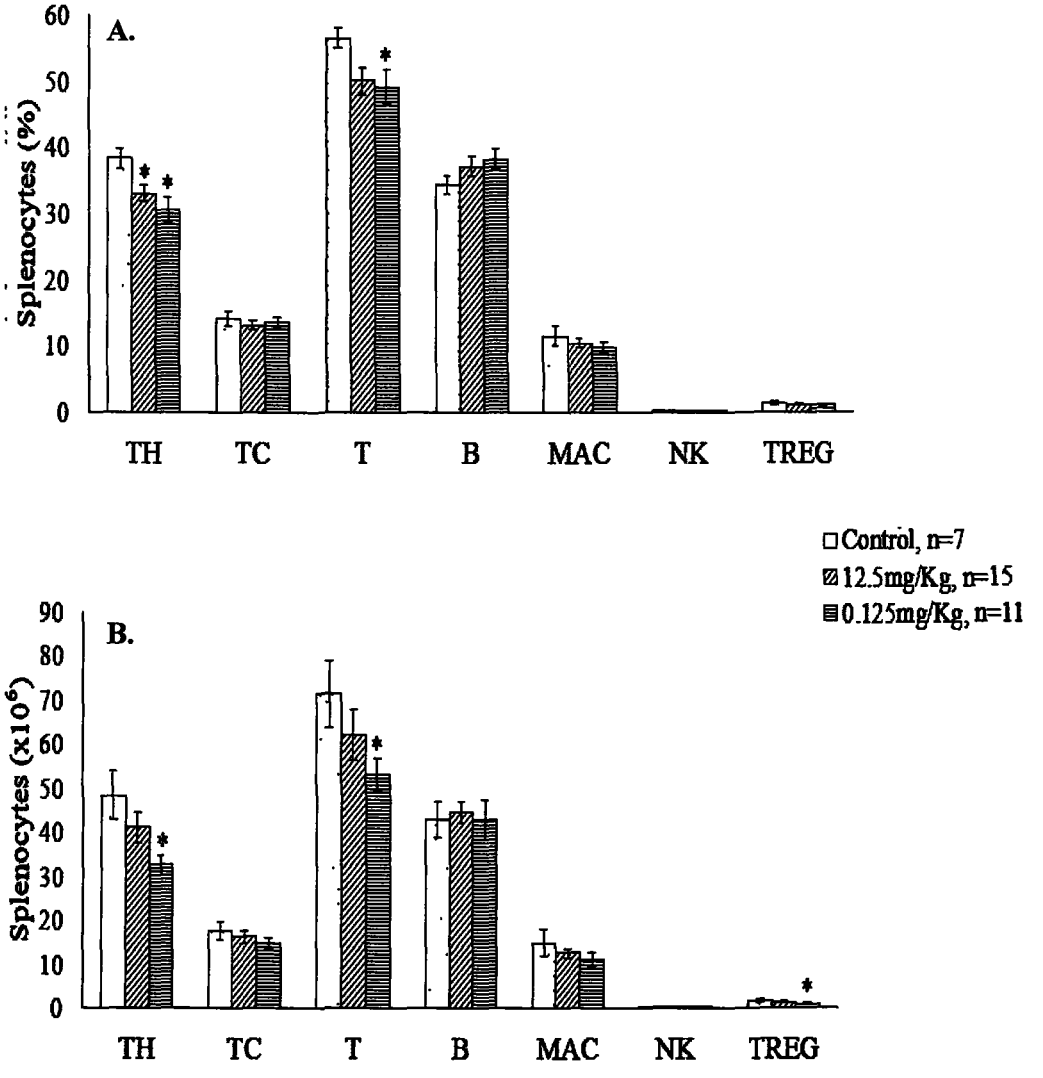

Figure 18. Immunophenotyping of NOD splenocytes after chronic exposure to PCB-153, up to 16-weeks. **(A)** Percentages; and **(B)** Total numbers ($x10^6$) of splenic CD4 (TH), CD8 (TC), CD3 (total T-cells; T), CD45RB220 (B-cells; B), CD11b (MAC), NK1.1 (NK), and CD4+CD25+ (TREG) cells. Female NOD mice were treated with PCB-153 as outlined in the legend of Figure 17. Splenocytes were stained with antibodies against various cell markers and analyzed by flow cytometry. Data are presented as mean ± SEM with significance determined by student's T-test (*$p<0.05$).

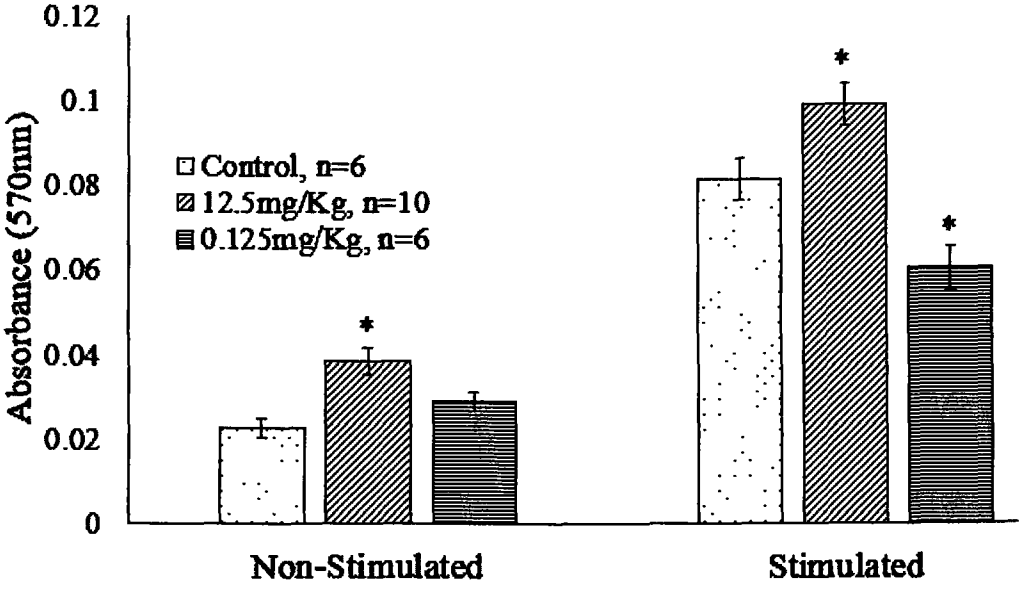

Figure 19. Proliferation of T-cells obtained from female NOD mice after chronic exposure to PCB-153, up to 16-weeks. Female NOD mice were treated as outlined in Figure 17. Splenocytes were cultured in the absence (non-stimulated) or presence (stimulated) of ConA (3μg/mL) for 72 hours. Proliferation of T-cells was measured by use of the Alamar Blue colorimetric assay, and absorbance determined at 570 nm. Data are presented as mean ± SEM with significance determined by student's T-test (*$p<0.05$).

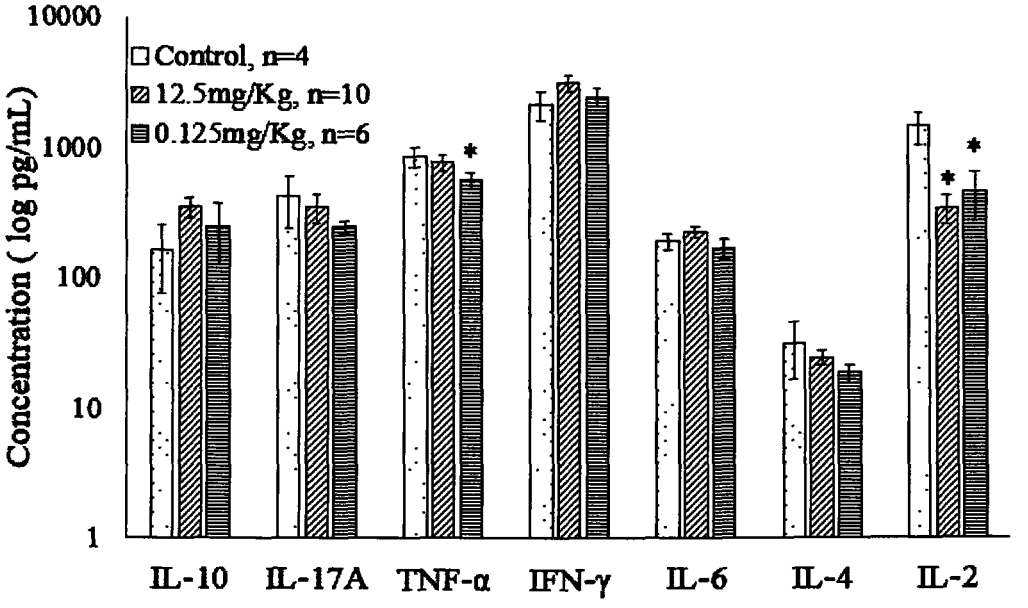

Figure 20. Quantification of cytokine levels after chronic exposure of NOD females to PCB-153, up to 16-weeks. Cytokines acquired from supernatants of splenocytes obtained from mice exposed to 0.125 or 12.5 PCB-153 mg/Kg as outlined in the legend of Figure 17. After 48 h of culture in the presence of ConA (3μg/mL), supernatants were analyzed using a CBA mouse $T_{H1}/T_{H2}/T_{H17}$ kit that permits quantification of IL-6, TNF-α, IFN-γ, IL-2, IL-4, IL-10 and IL-17A. Data shown are mean ± SEM; with significance determined by student's T-test (*$p<0.05$).

Chapter VI

DISCUSSION

To the best of our knowledge, this study has investigated for the first time the effect of a persistent organic pollutant (POP) polychlorinated biphenyl (PCB), PCB-153, on the development of T1D in an experimental mouse model, showing that exposure of prediabetic NOD females to high (25 mg/kg/week, over the period of 16 weeks), as well as low (0.25 mg/kg/week) PCB-153 doses, decreases the incidence and prevalence of T1D. Given that this is the first time that the PCB congener's diabetogenic effect was evaluated, and that reports about possible effects of other congeners in the context of T1D do not exist, mouse studies reporting other persistent organic pollutants' role in the pathogenesis of autoimmune T1D can be used as a reference for a comparison. So far, only few POPs have been tested, with DDT's metabolite DDE exhibiting acceleration of disease (Cetkovic-Cvrlje et al., 2015), BPA acting on potentiation of insulitis lesion and modulating invading lymphocytes towards pathogenic subsets (Bodin et al., 2013; Bodin et al., 2014), and TCE not affecting the development of disease (Raven et al., 2005).

Out of 209 PCB congeners, we were focused on PCB-153 in this study, as PCB-153 is the most prevalent congener which concentration is often used as a

representative of total PCB concentration (ΣPCB) in the sera. Most studies use ΣPCBs, rather than single congeners; however, there is necessary to understand the individual effects of prevalent PCB congeners, and their respective mechanism of action. This is important because PCBs invoke varied biological effects via mechanisms specific to their category, dioxin-like (dl)-PCB or non-dioxin-like (non-dl)-PCB. For instance, dl-PCBs use the aryl hydrocarbon (Ah)-receptor for its biological effects, whereas the non-dl-PCBs are unable to bind to the Ah receptor (Denison et al., 2002; Timsit and Negishi, 2007). Therefore, the different families of PCBs are likely able to interact with varied cell and tissue types, independently from one another, evoking incongruent responses, such as, for example, killing of thymocytes with ortho-substituted PCBs, but not with other substituted congeners (Tan et al., 2003).

Doses for the acute and chronic PCB-153 exposures were determined based on previously published results. Table 1 shows various concentrations of PCB-153 in lipids, sera, and breast milk of humans based on epidemiologic data, in parts per billion (PPB). Table 2 shows the cumulative doses (mg/Kg) of animal model studies, route of exposure, and animal model. As can be seen in both tables, the doses chosen for the acute and chronic PCB-153 exposures in this study fall within the range of other doses used in previous animal studies. For example, Smialowicz et al., 1997 administered single-oral doses of PCB-153 (3.58, 35.8, or 358mg/Kg) to B6C3F1 mice to determine the effects of the compound on antibody responses to sheep erythrocytes. Cumulatively, the doses chosen for our acute exposures in the

low- and high-dose, as well as our chronic exposure low-dose range, do not exceed those used in the aforementioned study; whereas our chronic exposure high-dose only marginally exceeds that of the highest dose used in the same study. In the context of human-exposure relevance, the high-doses chosen do not directly relate to all human concentrations observed (Table 1). Whereas the concentration of PCB-153 in sera of mice was not identified in this study, it was found that Sprague-Dawley rats exposed to 100mg/Kg PCB-153 at the endpoint of 14-weeks had an average concentration of 53.37ng/mL PCB-153 in the sera (NTP, 2006). Extrapolating this value, based on exposure amount and duration, serum levels of PCB-153 in our low-dose chronic exposure group would be about 27ng/mL (or 27 PPB), a value comparable to that observed in various upper-percentiles of epidemiologic studies (Table 1). The 16-week cumulative low-dose in our study was 4mg/Kg, which correlates to 4,000 PPB. Assuming that all administered PCB-153 stayed in the body of our mice, the cumulative dose of 4,000 PPB PCB-153 is comparable to the lipid concentration of PCB found in the highest percentile patients in Korrick et al., 2011.

Since T1D is a T-cell-dependent autoimmune disorder, we performed in vitro studies to determine whether PCB-153 can affect T-cell function. The exposure to high-doses of PCB-153 evoked suppression of T-cell proliferative responses, primarily through induction of apoptosis. Mori et al., 2006 found significantly decreased proliferative ability of T-lymphocytes from B6C3F1 mice, when cultured with mixtures of PCBs including PCB-153 in both high

(0.005μg/mL) and low (0.0005μg/mL) concentrations; however, PCB-153 alone had no effect on T-cell proliferation at the tested doses. This gives important highlight to the potential additive effects various PCBs may have when cultured together, which would explain why suppression is not seen at very low-doses of PCB-153 alone, but is evident at higher doses, like the ones used in our study. The ability of PCB-153 to suppress function and kill T-cells is especially important when considering its role in pathogenesis of T1D. Similar results to ours, concerning apoptosis induction, were obtained with both PCB-153 and other PCBs in previous studies, where PCB-153 induced apoptosis in thymocytes (Tan et al., 2003); non-dioxin-like PCB-153 (Sanchez-Alonso et al., 2003) and PCB-52 (Hwang et al., 2001) in neuronal cells; and dioxin-like PCB-104 in human monocytes (Shin et al., 2000).

Further support for the immunosuppressive effects of PCB-153 in our study was obtained by acute exposure of NOD mice to five injections of high- and low-doses of the compound over a period of 10 days. Young prediabetic male mice were used, as the focus of these experiments was the effect of PCB-153 on the immune system in vivo, not T1D development. Since diabetes incidence and prevalence in NOD males is prominently delayed and reduced, respectively, compared to their female counterparts (50% incidence of T1D in females by week 9 and 0% incidence in males at the same time point), it allows studying solely immune parameters and avoiding the influence of metabolic changes that accompany diabetes occurrence. Mice treated with high dose (50mg/Kg) PCB-153

displayed significantly reduced total number of splenocytes compared to controls. This finding would suggest a systemic toxicity induced by administration of PCB-153 in such a high-dose, however, there was no indication of body weight loss during this 10-day exposure (data not shown), and immunophenotyping revealed a targeting of a particular cell type. An analysis of mitogen-induced T-cell responses confirmed prominently inhibited proliferation of cells obtained from high-dose-treated mice. Immunophenotypic analysis and cytokine profiling confirmed significantly reduced total number of CD4+ T cells and a reduced IL-2 secretion, respectively. At the same time, a decreased total number of B-cells, and increased macrophage population, have been observed as well. Putting together, these data suggest that high-dose PCB-153 exhibits immunosuppressive effects, especially on adaptive immunity. Interestingly, a low-dose PCB-153 treatment, being 100-times lower, exhibited an immunosuppressive capability in terms of reduction of percentages, as well as absolute counts of CD4+ T_H cells, while not affecting total splenic cell counts and proliferative responses of T-cells. Moreover, analysis of cytokine secretion showed a significant reduction of all cytokines studied, being in line with a prominent immunosuppressive capability. Whereas such a dramatic shift in cytokine secretion was not observed by high-dose PCB-153 treatment, one should keep in mind that significantly increased level of macrophage population and their cytokine products in those mice might compensate for deficient quantities of cytokines secreted by T-cells.

Reduced splenic cellularity observed in our experiment confirms previously shown results in harbor porpoises in which increased ΣPCB-contamination was correlated with reduced spleen size (Beineke et al., 2005). The reduced T-cell proliferation observed in our experiment is in agreement with previously published results about reduced mitogen-induced T- and B-cell responses in bluegill sunfish (Duffy-Whitenour 2010); and suppressed humoral immune responses in C57Bl/6 mice fed a diet of beluga whale blubber contaminated with ΣPCBs for 3 months (Fournier et al., 2000). The same study provided immunophenotyping data where no differences were observed in cell populations of PCB-exposed mice versus controls, except for slight increases in CD8+ and CD45+ T-cells. Also, no effects were seen in splenic cell cultures stimulated with either PHA, ConA, or LPS in any group. Whereas the amount of various PCBs in whale blubber was not studied, a previous study has shown that whale blubber from the St. Lawrence estuary (source of whale blubber in the aforementioned study) has a concentration of hexachlorobiphenyls (e.g. PCB-153) at about 28.1mg/Kg (Lapierre et al., 1999). The immunosuppressive ability of PCBs, specifically as it pertains to the adaptive immune system, was observed in humans in the Yusho (Japan) and Yu-Cheng (Taiwan) disease cohorts, exposed to a wide-range poisoning caused by PCB-contaminated rice bran oil. Given the vastness of the poisoning and the magnitude of those affected, individuals within those cohorts have been subject to several epidemiologic studies. Thus, Lu et al., 1985 provided data that point towards immunosuppression through affection of CD4+ T cells, similar to ones described in

our study. They observed a significantly reduced total T-cells, T-helper cells, and active T-cells in peripheral blood samples, though, without a reduction in ConA-induced T-cell proliferation. The same patients were analyzed three years later, and the total T-cell population returned to normal-levels, though T-cytotoxic cells were increased along with a continued decrease in T-helper cells. Furthermore, post-natal exposure of Dutch infants to PCBs resulted in significantly reduced monocyte counts in peripheral blood samples, especially with the di-ortho-substituted PCBs (e.g. PCB-153). However, at 18 months of age, the same cohort was shown to have significantly increased CD8+ cells, along with an increase in T-cells. Though, these findings give indication towards immunostimulation, the authors conclude that the increased presence of subclinical infections may have been the reason for the increase in CD8+ cells, essentially compensating for the observed decrease in monocytes and granulocytes (Weisglas-Kuperus, 2000). Opposite findings were described in infants prenatally exposed to PCB-153, where CD8+ T-cells decreased with increased compound concentration in sera (Glynn et al., 2008), though significance was lost after accounting for potential confounders. Daniel et al., 2001 analyzed blood sera collected from 146 patients for PCB concentration and an array of immune parameters, including lymphocyte subpopulations, lymphocyte function, as well as autoantibodies, were reported. A significant decrease of CD25+ lymphocytes, reduced lymphocyte response and reduced levels of autoantibodies were observed in patients exposed to high levels of PCB-153. Collectively, these results are in line with our observation about immunosuppressive ability of PCB-

153 in mice, and suggest that PCB-153 in higher concentrations is able to cause immunosuppression in humans.

Cytokine-wise, PCB-153, in contrast to dioxin-like PCB, PCB-118, was shown not to be able to induce TH1 or TH2 differentiation (Gaspar-Ramirez et al., 2012). Therefore our data are in line with that finding, as an increase in neither IFN-γ nor IL-4/IL-10 secretion was observed. Tharappel et al., 2001 found that rats exposed to PCB-153 had increased DNA binding activity of NF-κB as well as STAT3; also, a decrease in activity of STAT5 was seen, but did not reach statistical significance ($p=0.07$). This gives further indication to the potential immunosuppressive effects caused by PCB-153, since STAT3 and STAT5 are activated by various cytokines, including IL-6 and IL-10 (STAT3) and IL-2 (STAT5) (Lin and Leonard, 2000; Niemand et al., 2003).

Further support for the immunosuppressive capabilities of PCB-153 was shown in the reduced incidence of T1D in mice chronically treated with both a high (12.5mg/Kg)- and low (0.125mg/Kg)-dose of the compound. Across all endpoints (2-, 8-, and 16-weeks) CD4+ cells seemed to be the most affected cell population by PCB-153 exposure, with similar reductions reflected in all T-(CD3+) cells (at 8- and 16-weeks). Furthermore, PCB-153 at the low-dose reduced proliferative capacity of T-cells cells at all endpoints (2-, 8-, and 16-weeks). As seen in the acute treatment experiments, a dichotomy was apparent between the high- and low-dose groups: whereas the high-dose group showed trends and significant decreases in CD4+ cells across all endpoints, the unaffected/increase in proliferative responses

of T-cells exposed to the high-dose of PCB-153, despite the significant decrease in IL-2 secretion, was observed. This alone could be evidence that the T-lymphocytes are functionally unaltered or compensated in higher-dose exposures. Another explanation for non-suppressed proliferation of T-cells observed in high-dose exposure groups could be an increased susceptibility and a response to bacterial and/or viral infections, as previously suggested in other studies (Dallaire et al., 2006; Hall et al., 2006; Imanishi et al., 1980; Weisglas-Kuperus et al., 2000). The low-dose group, had a consistently significant decrease in TNF-α and IL-2 at both the 8- and 16-week endpoints.

The evidence from all endpoints shows that the classical paradigm of T1D pathogenesis, the polarization of the TH1/TH2 subsets (Sia, 2005), is not readily applicable to the case of PCB-153 exposures and immune system alterations. If a shift towards either TH1 or TH2 was occurring, it would likely be expected to see an increase in cytokines keystone to one cell type, with a decrease in cytokines observed in the other. This was not the case in this study. A decrease in TNF-α suggests a reduction in the pro-inflammatory response evoked by macrophages (primarily) and CD4+ cells (secondarily), (Chatzidakis and Mamalaki, 2007; Uno et al., 2007), which would further support the decreased incidence of T1D in the low-dose group. Lee et al., 2005 further supports this conclusion, discovering that neonatal NOD mice administered with TNF-α resulted in increased onset of T1D, whereas TNF-α blockade led to disease abatement, largely due to immunomodulation of specific dendritic cell subsets. A reduction in IL-2, a chief

cytokine responsible for the differentiation and maturation of T-cells, would indicate a reduction in activation of T-cells and their functionality (Hulme et al., 2012; Malek 2003. Interestingly, previous research has shown PCBs' capability of suppressing IL-2 production and secretion (Exon et al., 1985; Steppan et al., 1993), as well as reduced adaptive immune responses specific to T-cells (Duffy-Whritenour et al., 2010; Li et al., 2013; Lu et al., 1985; Tan et al., 2003). It is possible that calcium-modulation, through activation of calcium-and-calmodulin-dependent kinase II (CaM kinase II), may be the culprit of reduced IL-2 secretion in the mice chronically-exposed to PCB-153. It is known that activated CaM kinase II increases the proliferative function of T-cells along with suppression of their IL-2 secretion (Lin et al., 2005). Interestingly, Fischer et al., 1999 found that non-coplanar PCBs (e.g. PCB-153) were able to induce activation of CaM kinase II in a dose-dependent manner, leading to an increase in free intracellular calcium. Therefore, it seems highly feasible that activation of CaM kinase II by PCB-153 is the perpetrator of the increased T-cell proliferation and reduced IL-2 secretion in the chronically high-dose PCB-153-exposed mice.

The role of PCB-153 in pathogenesis of T1D via modulation of β-cell activity has not been studied here. Previous studies have shown non-coplanar (e.g. PCB-153) PCBs' ability to alter calcium release in certain cell types, such as pancreatic insulinoma cells (pancreatic tumor derived from β-cells), leading to an increase in insulin release. The extent to which insulin was released was both time- and dose-dependent, with regards to PCB administration, and has implications for

insulin-secretion from normal β-cells (Fischer et al., 1996; Fischer et al., 1999). Therefore, a potential increase in insulin secretion from pancreatic β-cells, induced by administration of PCB-153, might contribute to the observed decrease in T1D incidence in exposed mice.

There are several epidemiologic studies that linked exposure to PCBs with development of autoimmunity. An increased risk of self-reported rheumatoid arthritis was described in women with increased ΣPCB concentration in blood (Lee et al., 2007), as well as systemic lupus erythematosus in women of the Yu-Cheng cohort (Tsai et al., 2007), while a higher frequency of anti-GAD antibodies in PCB-exposed factory workers suggested a positive association of ΣPCB and T1D (Langer et al., 2002). Since the incidence of disease was not observed in this study, conclusions about the effects of PCB on T1D development could not be drawn. Another epidemiologic study, that followed individuals of the Yu-Cheng cohort found a decreased incidence of diabetes mellitus in males exposed to ΣPCBs (Li et al., 2013). However, this study did not specify the type of diabetes, so one cannot distinguish between T1D and T2D occurrence. Rignell-Hydborn et al., 2010 investigated the role of PCB-153 and DDE in T1D and concluded that these compounds do not exhibit negative effects on T1D development, even implicating a possible protection. Seemingly disparate from this one-and-only epidemiologic study correlating directly PCBs and T1D (Rignell-Hydborn et al., 2010), recent experimental data (Cetkovic-Cvrlje et al., 2015), in conjecture with the herein shown results, may provide further insight. Cetkovic-Cvrlje et al., 2015 showed

that DDE was able to accelerate T1D development in NOD mice treated chronically with the compound, in an experimental design very similar to the one presented in this manuscript. Given the prominent immunosuppressive antidiabetogenic effects of PCB-153, as evidenced by the in vitro and in vivo studies presented herein, it is possible that they might be able to mask the diabetogenic action of DDE. Further experimental studies will be needed to support this hypothesis.

Table 1: Sera and lipid (TEQ) concentrations of PCB-153 (PPB) from literature review.

PPB Low	PPB Median[b] or Mean[c]	PPB High	Route	Source	Individual	Publication
0.08	0.39[b]	5.9	Blood	Blood	Pediatric**	Weisglas-Kuperus et al., 2000
0.08	0.4[b]	2.08	Blood	Cord	Neonatal*	Weisglas-Kuperus et al., 2000
0.389	1.0251[c]	2.221	Blood	Blood	Adult****	Daniel et al., 2001
0.59	2.07[b]	7.35	Blood	Blood	Adult****	Weisglas-Kuperus et al., 2000
0.1	2.4[b]	11.4	Blood	Blood	Adult****	Rignell-Hydbom et al., 2010
4.224	6.659[b]	9.094	TEQ-Lipid	Lipid	Neonatal*	Weisglas-Kuperus et al., 1995
4.9	13.9[c]	73.6	Blood	Lipid	Adolescent***	Leijs et al., 2009
23	60[b]	179	Blood	Lipid	Adult****	Glynn et al., 2008
0	187[b]	853	Milk	Breast	Adult****	Glynn et al., 2008
164	250[c]	4248	Blood	Lipid	Pediatric**	Korrick et al., 2011

Neonatal* is defined as 0-18 months of age; Pediatric** is defined as 18.1-13 years of age; Adolescent*** is defined as 13.1-17.9 years of age; Adult**** is defined as 18 years and older.

Table 2: Cumulative doses of PCB-153 (mg/Kg) used in various animal models.

Dose Amount (mg/Kg)	Cumulative Dose (mg/Kg)	# of Treatments	Organism	Route	Publication
0.04	0.14	4	Rat	I.P.	Tharappel et al., 2002
0.18	0.18	1	Rat	I.P.	Parkinson et al., 1983
0.04	0.22	6	Mouse	I.P.	Lu, 2004
0.11	0.43	4	Rat	I.P.	Tharappel et al., 2002
0.11	2.16	20	Mouse	I.P.	Strathmann et al., 2006
3.58	3.58	1	Mouse	Oral	Smialowicz, 1997
5.00	5.00	1	Bluegill Sunfish	I.P.	Duffy-Whritenour et al., 2010
4.00	20.00	5	Rat	I.P.	Liu et al., 2014
35.80	35.80	1	Mouse	Oral	Smialowicz et al., 1997
50.00	50.00	1	Bluegill Sunfish	I.P.	Duffy-Whritenour et al., 2010
25.00	50.00	2	Rat	I.P.	Desaulniers et al., 1998
16.00	80.00	5	Rat	I.P.	Liu et al., 2014
100.00	100.00	1	Mouse	I.P.	Ropstad et al., 2006
32.00	160.00	5	Rat	I.P.	Liu et al., 2014
358.00	358.00	1	Mouse	Oral	Smialowicz et al., 1997
0.50	2.50	5	Mouse	I.P.	Current study
0.125	4.00	32	Mouse	I.P.	Current study
50.00	250.00	5	Mouse	I.P.	Current study
12.50	400.00	32	Mouse	I.P.	Current study

Chapter VII

CONCLUSIONS

The study presented herein shows for the first time the effects of the non-dioxin-like, non-coplanar PCB-153 on incidence of T1D in an animal model, with decreased disease incidence being observed in both the high- and low-dose treatment groups of chronically treated NOD mice. The observed reduction of T1D incidence is attributed to the significant decrease in a particular CD4+ TH1-type T-cell subset of the adaptive immune system and its IL-2 cytokine secretion. Support for these findings is provided by the acute PCB-153 exposure experiments using NOD males, where the immune parameters analyzed showed significant reduction of the same CD4+ T-cell subset and IL-2, along with reduced T-cell functionality. These results bridge the existing knowledge gap regarding the association of non-dioxin-like PCBs and T1D, not only showing that PCB-153 indeed affects diabetogenesis through induction of immunosuppression, but bringing awareness regarding the potential effects of PCB-153 on other co-existing pollutants. Effects and potential co-kinetics of various POPs in the development of T1D in animal models should be analyzed, to define a causation and provide understanding of epidemiologic data.

REFERENCES

REFERENCES

Agency for Toxic Substances and Disease Registry (ATSDR). 2000. Toxicological Profile for Polychlorinated Biphenyls (PCBs). Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

Ahlborg, U., Hanberg, A., & Kenne, K. 1992. Risk Assessment of Polychlorinated Biphenyls (PCBs). Institute of Environmental Medicine Karolinska Institutet, Stockholm, Sweden. Nord 1992:26.

Anderson, M. S., & Bluestone, J. A. 2005. The NOD Mouse: A Model of Immune Dysregulation. Annu. Rev. *Immunol.* 2005. 23:447-85. doi:10.1146/annurev.immunol.23.021704.115643

Baes, M., Gulick, T., Choi, H.S. et al. 1994. A New Orphan Member of the Nuclear Hormone Receptor Superfamily that Interacts with a Subset of Retinoic Acid Response Elements. *Molecular and Cellular Biology* 14:1544-1552.

Beineke, A., Siebert, U., McLachlan, M., Bruhn, R., Thron, K., Failing, K., Baumgartner, W., et al. 2005. *Environmental Science Technologies* 39:3933-3938.

Bitman, J., Cecil, H.C., & Harris, S.J. 1972. Biological Effects of Polychlorinated Biphenyls in Rats and Quail. *Environmental Health Perspectives* April 1972:145-149.

Bodin, J., Bolling, A.K., Becher, R., Kuper, F., Lovik, M., & Nygaard, U.C. 2014. Transmaternal Bisphenol A Exposure Accelerates Diabetes Type 1 Development in NOD Mice. *Toxicological Sciences* 137:311-323.

Bodin, J., Bolling, A.K., Samuelsen, M., Becher, R., Lovik, M., & Nygaard, U.C. 2013. Long-Term Bisphenol A Exposure Accelerates Insulitis Development in Diabetes. *Immunopharmacol. Immunotoxicol.* 35:349-358.

Canesi, L., Ciacci, C., Betti, M., Scarpato, A., Citterio, B., Pruzzo, C., & Gallo, G. 2003. Effects of PCB Congeners on the Immune Function of *Mytilus* Hemocytes: Alterations of Tyrosine Kinase-Mediated Cell Signaling. *Aquatic Toxicology* 63:293-306.

Cárdenas-González, M., Gaspar-Ramírez, O., Pérez-Vázquez, F.J., et al. 2013. p,p'-DDE, a DDT Metabolite, Induces Proinflammatory Molecules in Human Peripheral Blood Mononuclear Cells "*in vitro*". *Exp. Toxicol. Pathol.* 65:661-665.

Česko-Slovenská Pediatrie. Prvni číslo 1.4.1946 pod názvem Pediatrické listy. 2007.

Cetkovic-Cvrlje, M., Dragt, A.L., Vassiley, A., Liu, X.P., & Uckun, F.M. 2003. Targeting JAK3 with JANEX-1 for Prevention of Autoimmune Type 1 Diabetes in NOD Mice. *Clinical Immunology* 106:213-225.

Cetkovic-Cvrlje, M., Gerling, I.C., Muir, A., Atkinson, M.A., Elliot, J.F., & Leiter, E.H. 1997. Retardation or Acceleration of Diabetes in NOD/Lt Mice Mediated by Intra-Thymic Administration of Candidate β Cell Antigens. *Diabetes* 46:1975-1982.

Cetkovic-Cvrlje, M., Olson, M., & Ghate, K. 2012. Targeting Janus Tyrosine Kinase (JAK)3 with Inhibitor Induces Secretion of TGFβ by $CD4^+$ T-cells. *Cellular & Molecular Immunology* 9:350-360.

Cetkovic-Cvrlje, M., Olson, M., Schindler, B., & Gong, H.K. 2015. Exposure to DDT Metabolite p,p'-DDE Increases Autoimmune Type 1 Diabetes Incidence in NOD Mouse Model. *J. Immunotoxicology.* Manuscript submitted for publication.

Cetkovic-Cvrlje, M., Roers, B.A., Schonhoff, D., Waurzyniak, B., Liu, X. P., & Uckun, F.M. 2002. Treatment of Post-Bone Marrow Transplantation Acute Graft-vs. Host Disease (GVHD) with a Rationally Designed JAK3 Inhibitor. *Leukemia Lymphoma* 43:1447-1453.

Cetkovic-Cvrlje, M., Roers, B.A., Waurzyniak, B., Liu, X.P., & Uckun, F.M. 2001. Targeting Janus Kinase 3 to Attenuate the Severity of Acute Graft-Versus-Host Disease across the Major Histocompatibility Barrier in Mice. *Blood* 98:1607-1613.

Cetkovic-Cvrlje, M. & Uckun, F.M. 2004. Dual Targeting of Bruton's Tyrosine Kinase and Janus Kinase 3 with Rationally Designed Inhibitors Prevents Graft-Versus-Host Disease (GVHD) in a Murine Allogeneic Bone Marrow Transplantation Model. *British Journal of Haematology* 126:821-827.

Cetkovic-Cvrlje, M. & Uckun, F.M. 2005. Effect of Targeted Disruption of Signal Transducer and Activator of Transcription (Stat) 4 and Stat6 genes on the

Autoimmune Diabetes Development Induced by Multiple Low Doses of Streptozotocin. *Clinical Immunology* 114:299-306.

Chatzidakis, I. & Mamalaki, C. 2010. T Cells as Sources and Targets of TNF: Implications for Immunity and Autoimmunity. *Curr. Dir. Autoimmun.* 11:105-118.

Cnop, M., Welsh, N., Jonas, J.C., Jorns, A., Lenzen, S., & Eizirik, D.L. 2005. Mechanisms of Pancreatic β-Cell Death in Type 1 and Type 2 Diabetes. *Diabetes* 54 (Suppl. 2):S97-S107.

Curran, C.P., Nebert, D.W., Genter, M.B., et al. 2011. *In utero* and Lactational Exposure to PCBs in Mice: Adult Offspring Show Altered Learning and Memory Depending on *Cyp1a2* and *Ahr* genotypes. *Environ. Health Perspect.* 119:1286-1293.

Dabelea, D., Mayer-Davis, E.J., Saydah, S., et al. 2014. Prevalence of Type 1 and Type 2 Diabetes among Children and Adolescents from 2001 to 2009. *JAMA*, 311:1778-1786.

Dallaire, F., Dewailly, E., Vezina, C., et al. 2006. Effect of Prenatal Exposure to Polychlorinated Biphenyls on Incidence of Acute Respiratory Infections in Preschool Inuit Children. *Environ. Health Perspect.* 114:1301-1305.

Daniel, V., Huber, W., Bauer, K., Suesal, C., Conradt, C., & Opelz, G. 2001. Associations of Blood Levels of PCB, HCHs, and HCB with Numbers of Lymphocyte Subpopulations, in Vitro Lymphocyte Response, Plasma Cytokine Levels, and Immunoglobulin Autoantibodies. *Environmental Health Perspectives* 109:173-178.

Delovitch, T.L. & Singh, B. 1997. The Nonobese Diabetic Mouse as a Model of Autoimmune Diabetes: Immune Dysregulation Gets the NOD. *Immunity* 7:727-738.

Den Hond, E., Roels, H.A., Hoppenbrouwers, K., et al. 2002. Sexual Maturation in Relation to Polychlorinated Aromatic Hydrocarbons: Sharpe and Skakkebaek's Hypothesis revisited. *Environmental Health Perspectives* 110:771-776.

Denison, M.S., Pandini, A., Nagy, S.R., Baldwin, E.P., & Bonati, L. 2002. Ligand Binding and Activation of the Ah Receptor. *Chemico-Biological Interactions* 141:3-24.

Desaulniers, D., Leingartner, K., Wade, M., Fintelman, E., Yagminas, A., & Foster, W.G. 1999. Effects of Acute Exposure to PCBs 126 and 153 on Anterior Pituitary and Thyroid Hormones and FSH Isoforms in Adult Sprague Dawley Male Rats. *Toxicological Sciences* 47:158-169.

Dickerson, S.M., Cunningham, S.L., Patisaul, H.B., Woller, M.J. & Gore, A.C. 2011. Endocrine Disruption of Brain Sexual Differentiation by Developmental PCB Exposure. *Endocrinology* 152:581-594.

Duffy-Whritenour, J.E., Kurtzman, R.Z., Kennedy, S., & Zelikoff, J.T. 2010. Non-coplanar Polychlorinated Biphenyl (PCB)-Induced Immunotoxicity is Coincident with Alterations in the Serotonergic System. *Journal of Immunotoxicology* 7:318-326.

EMEP/CORINAIR Guidebook. 2005. Sources of PCB Emissions. Accessed August 28, 2013. http://www.eea.europa.eu.

EPA. 2009. Persistent Organic Pollutants: A Global Issue, a Global Response. http://www.epa.gov/oia/toxics/pop.html accessed on 08/24/13.

EPA. 2012. Health Consultation: Technical Support Document for a Polychlorinated Biphenyl Reference Dose (RfD) as a Basis for Fish Consumption Screening Values (FCSVs).

Exon, J.H., Talcott, P.A., & Kroller, L.D. 1985. Effect of Lead, PolychlorinatedBiphenyls, and Cyclophosphamide on Rat Natural Killer Cells, Interleukin 2, and Antibody Synthesis. *Fund. Appl. Toxicol.* 5:158:164.

Fischer, L.J., Wagner, M.A., & Madhukar, B.V. 1999. Potential involvement of calcium, CaM kinase II, and MAP kinases in PCB-stimulated insulin release from RINm5F cells. *Toxicol. Appl. Pharmacol.* 159:194-203.

Fischer, L.J. Zhou, H.R., & Wagner, M.A. 1996. Polychlorinated biphenyls releaseinsulin from RINm5F cells. *Life Sci.* 59:2041-2049.

Fournier, M., Degas, V., Colborn, T., Omara, F.O., Denizeau, F., Potworowski, E.F., & Brousseau, P. 2000. Immunosuppression in Mice Fed on Diets Containing Beluga Whale Blubber from the St. Lawrence Estuary and the Arctic Populations. *Toxicology Letters* 112-113:311-317.

Fusani, L., Seta, D.D., Dessi-Fulgheri, F., & Farabollini, F. 2007. Altered Reproductive Success in Rat Pairs After Environmental-Like Exposure to Xenoestrogen. *Proc. R. Soc. B.* 274:1631-1636.

Gaspar-Ramirez, O., Perez-Vasquez, F.J., Pruneda-Alvarez, L.G., Orta-Garcia, S.T., Gonzalez-Amaro, R., & Perez-Maldonado, I.N. 2012. Effect of Polychlorinated Biphenyls 118 and 153 on Th1/Th2 Differentiation. *Immunopharmacology and Immunotoxicology* 34:627-632.

Glynn, A., Thuvander, A., Aune, M., Johannisson, A., Darnerud, P.O., Ronquist, G., & Cnattingius, S. 2008. Immune Cell Counts and Risk of Respiratory Infections Among Infants Exposed Pre-and Postnatally to Organochlorine Compounds: A Prospective Study. *Environmental Health.* doi:10.1186/1476-069X-7-62

Govarts, E., Nieuwenhuijsen, M., Schoeters, G., et al. 2012. Birth Weight and Prenatal Exposure to Polychlorinated Biphenyls (PCBs) and Dichlorodiphenyldichloroethylene (DDE): A Meta-Analysis Within 12 European Birth Cohorts. *Environ. Health Perspect.* 120:162-170.

Grunnet, L.G. & Mandrup-Poulsen, T. 2011. Cytokines and Type 1 Diabetes: A Numbers Game. *Diabetes* 60:697-699.

Guo, Y.L., Lambart, G.H., Hsu, C.C., & Hsu, M.M. 2004. Yucheng: Health Effects of Prenatal Exposure to Polychlorinated Biphenyls and Dibenzofurans. *In. Arch. Occup. Environ. Health* 77:153-158.

Hall, A.J., Hugunin, K., Deaville, R., Law, R.J., Allchin, C.R., & Jepson, P.D. 2006. The Risk of Infection from Polychlorinated Biphenyl Exposure in the Harbor Porpoise (Phocoena phocoena): A Case-Control Approach. *Environ. Health Perspect.* 114:704-711.

Hulme, M.A., Wasserfall, C.H., Atkinson, M.A., & Brusko, T.M. 2012. Central Role for Interleukin-2 in Type 1 Diabetes. *Diabetes.*61:14-22.

Hwang, S.G., Lee, H.C., Lee, D.W., et al. 2001. Induction of Apoptotic Cell Death by a p53-Independent Pathway in Neuronal SK-N-MC Cells After Treatment with 2,2',5,5'-Tetrachlorobiphenyl. *Toxicology* 165:179-188.

Imanishi, J., Nomura, H., Matsubara, M., et al. 1980. Effect of Polychlorinated Biphenyl on Viral Infections in Mice. *Infection and Immunity* 29:275-277.

Iqbal, N. 2007. The Burden of Type 2 Diabetes: Strategies to Prevent or Delay Onset. *Vasc. Health Risk Manag.* 3:511-520.

Johansen, E.B., Knoff, M., Fonnum, F., et al. 2011. Postnatal Exposure to PCB 153 and PCB 180, but not to PCB 52, Produces Changes in Activity Level and

Stimulus Control in Outbred Male Wistar Kyoto Rats. *Behavioral and Brain Functions* 7:18. http://www.behavioralandbrainfunctions. com/content/7/1/18.

Jun, H.S., Yoon, C.S., Zbtnuik, L., van Rooijen, N., Yoon, J.W. 1999. The Role of Macrophages in T Cell-Mediated Autoimmune Diabetes in Nonobese Diabetic Mice. *J. Exp. Med.* 189:347-358.

Juvenile Diabetes Research Foundation (JDRF). 2014. http://jdrf.org/about-jdrf/factsheets/type1-diabetes-facts/ accessed on 01/20/2014.

Kanagawa, Y., Matsumoto, S., Koike, S., et al. 2008. Association of Clinical Findings in Yusho Patients with Serum Concentrations of Polychlorinated Biphenyls, Polychlorinated Quarterphenyls and 2, 3, 4, 5, 8-Pentachlorodibenzofuran more than 30 Years After the Poisoning Event. *Environmental Health.* doi:10.1186/1476-069X-7-47

Kikutani, H. & Makino, S. 1992. The Murine Autoimmune Diabetes Model: NOD and Related Strains. *Advances in Immunology* 51:285-322.

Knip, M. & Simell, O. 2012. Environmental Triggers of Type 1 Diabetes. *Cold Spring Harb. Perspect. Med.* 2:a007690.

Kobayahsi, K., Miyagawa, M., Wang, R.S., Suda, M., Sekiguchi, S., & Honma, T. 2009. Effects of *in Utero* Exposure to 2,2, 4,4', 5,5'-Hexachlorobiphenyl on Postnatal Development and Thyroid Function in Rat Offspring. *Industrial Health* 47:189-197.

Koplan, J P. 2000. Toxicological Profile For Polychlorinated Biphenyls (PCBs).U.S. Department of Human Health Services: Public Health Service Agency for Toxic Substances and Disease Registry November 2000.

Korrick, S.A., Lee, M.M., Williams, P.L., et al. 2011. Dioxin Exposure and Age of Pubertal Onset Among Russian Boys. *Environ. Health Perspect.* 119:1339 1344.

Krogenaes, A K., Nafstad, I., Skare, J U., Farstad W, & Hafne A. 1998. In Vitro Reproductive Toxicity of Polychlorinated Biphenyl Congeners 153 and 126. *Reproductive Toxicology* 12:575-580.

Langer, P., Tajtakova, M., Guretzki, H.J., Kocan, A., Petrik, J., Chovancova, J., Drobna, B., Jursa, S., Pavuik, M., Tmovic, T., Sebokova, E., & Klimes, I. 2002. High Prevalence of Anti-Glutamic Acid Decarboxylase (anti-GAD)

Antibodies in Employees at a Polychlorinated Biphenyl Production Factory. *Archives of Environmental Health* 57:412-415.

Lapierre, P., De Guise, S., Muir, D.C.G., Norstrom, R., Beland, P, & Fournier, M. 1999. Immune Functions in the Fisher Rat Fed Beluga Whale (*Delphinapterus leucas*) Blubber from the Contaminated St. Lawrence Estuary. *Environmental Research Section A* 80:S104-S112.

Lawrence, J.M., Imperatore, G., Dabelea, D., et al. 2014. Trends in Incidence of Type 1 Diabetes Among Non-Hispanic White Youth in the U.S., 2002-2009. *Diabetes*, 63:3938-3945.

Lee, D., Lee, I., Song, K., Steffes, M., Toscano, W., Bake, B A., & Jacobs, D R. 2006. A Strong Dose-Response Relation Between Serum Concentrations of Persistent Organic Pollutants and Diabetes: Results from the National Health Examination Survey 1999-2002. *Diabetes Care* 29:1638-1644.

Lee, D., Steffes, M., & Jacobs, D.R. 2007. Positive Associations of Serum Concentration of Polychlorinated Biphenyls and Organochlorine Pesticides with Self-Reported Arthritis; Especially Rheumatoid Type, in Women. *Environmental Health Perspectives* 115:883-888.

Lee, L.F., Xu, B., Michle, S.A., et al. 2005. The Role of TNF-α in the Pathogenesis of Type 1 Diabetes in the Nonobese Diabetic Mouse: Analysis of Dendritic Cell Maturation. *PNAS* 102:15995-16000.

Lehmann, J.M., McKee, D.D., Watson, M.A., Willison, T.M., Moore, J.T., & Kliewer, S.A. 1998. The Human Orphan Nuclear Receptor PXR is Activated by Compounds that Regulate *CYP3A4* Gene Expression and Cause Drug Interactions. *Journal of Clinical Investigations* 102:1016-1023.

Leijs, M.M., Koppe, J.G., Olie, K., Van Aalderen, M.C., De Voogt, P., & Ten Tusscher, G.W. 2009. Effects of Dioxins, PCBs, and PBDEs on Immunology and Hematology in Adolescents. *Environ. Sci. Technol.* 43:7946-7951.

Leiter, E.H. 1990. The NOD Mouse Meets the 'Nerup Hypothesis': Is Diabetogenesis the Result of a Collection of Common Alleles Present in Unfavorable Combinations? *Frontiers of Diabetes Research: Lessons from Animal Diabetes* (Dr. Eleazar Shafrir, Ed.) 3:54-58.

Leiter, E.H. 1993. The NOD Mouse: A Model for Analyzing the Interplay Between Heredity and Environment in Development of Autoimmune Disease. *Models of Type 1 Diabetes-Part One* 35:4-14.

Leiter, E.H. & Serreze, D.V. 1992. Antigen Presenting Cells and the Immunogenetics of Autoimmune Diabetes in NOD Mice. *Regional Immunology* 3:64-70.

Li, M.H., Tsai, P., Hseih, C., Guo, Y.L., & Rogan, W.J. 2013. Mortality After Exposure to Polychlorinated Dibenzofurans: 30 Years After the "Yucheng Accident". *Environ Res.* 120:71-75.

Li, M.H., Zhao, Y.D., & Hansen, L.G. 1994. Multiple Dose Toxicokinetic Influence on the Estrogenicity of 2, 2'4, 4', 5,5'-Hexachlorobiphenyl. *Bull. Environ. Contam. Toxicol.* 53:583-590.

Lin, J.X. & Leonard, W.J. 2000. The Role of STAT5a and STAT5b in Signaling by IL-2 Family Cytokines. *Oncogene* 19:2566-2576.

Lin, M.Y., Zal, T., Ch'en, I.L., Gascoigne, N.R.J., & Hedrick, S.M. 2005. A pivotal role for the multifunctional calcium/calmodulin-dependent protein kinase II in T Cells: from activation to unresponsiveness. *J. Immunology*. 174:5583-5592.

Liu, C., Yang, J., Fu, W., et al. 2014. Coactivation of the PI3/Akt and ERK Signaling Pathways in PCB153-Induced NF-κβ Activation and Caspase Inhibition. *Toxicology and Applied Pharmacology* http://dx.doi.org/10.1016/j.taap.2014.03.027.

Longnecker, M.P., & Daniels, J.L. 2001. Environmental Contaminants as Etiologic Factors for Diabetes. *Environ Health Perspect* 19:871-876.

Lu, Y.C. & Wu, Y.C. 1985. Clinical Findings and Immunological Abnormalities in Yu Cheng Patients. *Environ. Health Perspect.* 59:17-29.

Lu, Z., Lee, E., Robertson, L.W., Glauert H P., & Spear B T. 2004. Effect of 2,20,4,40,5,50-Hexachlorobiphenyl (PCB-153) on Hepatocyte Proliferation and Apoptosis in Mice Deficient in the p50 Subunit of the Transcription Factor NF kB. *Toxicological Sciences* 81:35-42. doi:10.1093/toxsci/kfh193 Advance Access publication June 16, 2004.

Lund, T., O'Reilly, L., Hutchings, P., et al. 1990. Prevention of Insulin-Dependent Diabetes Mellitus in Non-Obese Diabetic Mice by Transgenes Encoding Modified I-A β-Chain or Normal I-E α-Chain. *Nature* 345:727-729.

Malek, T.R. 2003. The Main Function of IL-2 is to Promote the Development of T Regulatory Cells. *J. Leukocyte Biology* 74:961-965.

Mori, C., Morsey, B., Levin, M., Nambiar, P.R., & De Guise, S. 2006. Immunomodulatory Effects of In Vitro Exposure to Organochlorines on T-Cell Proliferation in Marine Mammals and Mice. *J. Toxicol. And Environ. Health.*69:283-302.

Muller, A., Schott-Ohly, P., Dohle, C., & Gleichmann, H. 2002. Differential Regulation of T_H1- and T_H2-type Cytokine Profiles in Pancreatic Islets of C57BL/6 and BALB/c Mice by Multiple Low Doses of Streptozotocin. *Immunobiology* 205:35-50.

National Toxicology Program (NTP). 2006. NTP Technical Report on the Toxicology and Carcinogenesis Studies of 2,2',4,4',5,5' Hexachlorobiphenyl (PCB 153) (CAS No. 35065-27-1) in Female Harlan Sprague-Dawley Rats (Gavage Studies). *Natl. Toxicol. Program Tech. Rep. Ser.* 529:4-168.

Niemand, C., Nimmesgern, A., Haan, S., et al. 2003. Activation of STAT3 by IL-6 and IL-10 in Primary Human Macrophages is Differentially Modulated by Suppressor of Cytokine Signaling 3. *J. Immunotoxicol.* 170:3263-3272.

Noble, J.A. & Erlich, H.A. 2012. Genetics and Type 1 Diabetes. *Cold Spring Harb Perspect Med*, 2:a007732.

Notkins, A.L. 2002. Immunologic and Genetic Factors in Type 1 Diabetes. *J. Biological Chemistry* 277:42545-43548.

Okada, H., Kuhn, C., Feillet, H., & Bach, J-F. 2010. The 'Hygiene Hypothesis' for Autoimmune and Allergic Diseases: An Update. *Clin. Exp. Immunol.* 160:1-9.

Park, S.O., Cho, Y.R., Kim, H.J., et al. 2005. Unraveling the Temporal Pattern of Diet- Induced Insulin Resistance in Individual Organs and Cardiac Dysfunction in C57BL/6 Mice. *Diabetes* 54:3530-3540.

Parkinson, A., Safe, S.H., Robertson, L.W. et al, 1983. Immunochemical Quantitation of Cytochrome P-450 Isozymes and Epoxide Hydrolase in Liver Microsomes from Polychlorinated or Polybrominated Biphenyl-Treated Rats. *J. Biol. Chem.* 258:5967-5976.

Pinkse, G.G.M., Tysma, O.H.M., Bergen, C.A.M., et al. 2005. Autoreactive CD8 T Cells Associated with β Cell Destruction in Type 1 Diabetes. *PNAS* 102:18425-18430.

Pociot, F., & McDermott, M.F. 2002. Genetics of Type 1 Diabetes Mellitus. *Genes and Immunity*, 3:235-249.

Rabinovitch, A. 1998. An Update on Cytokines in the Pathogenesis of Insulin-Dependent Diabetes Mellitus. *Diabetes Metabolism. Reviews.* 14:129-151.

Raven, G., Christ, M., Perron-Lepage, M.F., Condevaux, F., & Descotes, J. 2005. Trichloroethylene Does Not Accelerate Autoimmune Diabetes in NOD Mice. *J. Immunotoxicol.* 1:141-148.

Rignell-Hydbom, A., Elfving, M., Ivarsson, S. A., et al. 2010. A Nested Case-Control Study of Intrauterine Exposure to Persistent Organo-Chlorine Pollutants in Relation to Risk of Type 1 Diabetes. *PLoS One* 5:e11281.

Ritter, L., Solomon, J., Stemeroff, M., & O'Leary, C. 1995. Persistent Organic Pollutants: An Assessment Report On: DDT-Aldrin-Dieldrin-Endrin-Chlordane-HeptachlorHexachlorobenzene-Mirex-Toxaphene-Polychlorinated Biphenyls-Dioxins and Furans. The International Programme on Chemical Safety (IPCS) within the framework of the Inter-Organization Programme for the Sound Management of Chemicals (IOMC).

Roberston, J.D., & Orrenius, S. 2000. Molecular Mechanisms of Apoptosis Induced by Cytotoxic Chemicals. *Crit. Rev. Toxicol.* 30:609-627.

Ropstad, E., Oskam, I.C., Lyche, J.L., et al. 2006. Endocrine Disruption Induced by Organochlorines (OCs): Field Studies and Experimental Models. *J. Toxicol. Environ. Health A.* 69:53-76.

Sakaguchi, S. 2000. Regulatory T-Cells: Key Controllers of Immunologic Self-Tolerance. *Cell* 101:455-458.

Sanchez-Alonso, J.A., Lopez-Aparicio, P., Recio, M.N., & Perez-Albarsanz, M.A. 2003. Apoptosis-Mediated Neurotoxic Potential of a Planar (PCB 77) and a Nonplanar (PCB 153) Polychlorinated Biphenyl Congeners in Neuronal Cell Cultures. *Toxicology Letters* 144:337-349.

Schatteman, G., Hanlon, H., Jiao, C., Dodds, S., & Christy, B. 2000. Blood-derived Angioblasts Accelerate Blood-Flow Restoration in Diabetic Mice. *J. Clin. Invest.*106:571-578.

Shafrir, E. 2007. Animal Models of Diabetes, Second Edition: Frontiers in Research. Dr. Eleazar Shafrir (Ed.) ISBN: 9780849395345.

Shin, K.J., Bae, S.S., Hwang, Y.A., Seo, J.K., Ryu, S.H., & Suh, P.G. 2000. 2, 2', 4, 6, 6' Pentachlorobiphenyl Induces Apoptosis in Human Monocytic Cells. *Toxicol. Appl. Pharmacol.* 169:1-7.

Silverstone, A.E., Rosenbaum, P.F., Weinstock, R.S., Bartell, S.M., Foushee, H.R., Shelton, C., & Pavuk, M. 2012. Polychlorinated Biphenyl (PCB) Exposure and Diabetes: Results from the Anniston Community Health Survey. *Environmental Health Perspectives* 120:726-732.

Sjödin, A., Schecter, A., Jones, R.S., Lee-Yang, W., Colacino, J.A., Malik-Bass, N., Zhang, Y., Anderson, S., McClure, C., Turner, W.E., & Calafat, A.M. 2014. Polybrominated Diphenyl Ethers, 2, 2', 4, 4',5, 5'-Hexachlorobiphenyl (PCB-153) and p,p'-Dichlorodiphenyldichloroethylene (p,p'-DDE) Concentrations in Sera Collected in 2009 Texas Children. *Environmental Science and Technology* just accepted manuscript.

Smialowicz, R.J., Devito, M.J., Riddle, M.M., Williams, & Birnbaum, L.S. 1997. Opposite Effects of 2,2',4,4',5,5'-Hexachlorobiphenyl and 2,3,7,8-Tetrachlorodibenozo-*p*-dioxin on the Antibody Response to Sheep Erythrocytes in Mice.

Smith, K.A. 1988. Interleukin-2: Inception, Impact, and Implications. *Science* 240:1169-1176.

Stenstrom, G., Gottsater, A., Bakhtadze, E., Berger, B., & Sundkvist, G. 2005. Latent Autoimmune Diabetes in Adults: Definition, Prevalence, Beta-cell Function, and Treatment. *Diabetes* 54 (Suppl. 2):S68-S72.

Steppan, L., DeKrey, G.K., Fowles, J.R., & Kerkvliet, N.I. 1993. Polychlorinated Biphenyl (PCB) Induced Alteration in the Cytokine Profile in the Peritoneal Cavity of Mice During the Course of P815 Tumor Rejection. *J. Immunol.* 150:134A.

Strathmann, J., Schwarz, M., Tharappel, J C., Glauert H P., Spear B T., Robertson LW., Appel K E., & Buchmann, A. 2006. PCB 153, a Non-dioxin–like Tumor Promoter, Selects for B-Catenin (Catnb)–Mutated Mouse Liver Tumors. *Toxicological Sciences* 93:34-40. doi:10.1093/toxsci/kfl041 Advance Access publication June 16, 2006.

Tan, Y., Li, D., & Song, R., Carpenter, DO. 2003. Ortho-Substituted PCBs Kill Thymocytes. *Toxicological Sciences* 76:328-337.

Ter Veld, M. G., Zawadzka, E., van den Berg, J. H., van der Saag, P. T., Rietjens, I. M., & Murk, A.J. 2008. Food-Associated Estrogenic Compounds Induce

Estrogen Receptor-Mediated Luciferase Gene Expression in Transgenic Male Mice. *Chemico-Biological Interactions* 174:126-133.

Tharappel, J.C., Lee, E.Y., Robertson, L.W., Spear, B.T., & Glauert, H.P. 2002. Regulation of Cell Proliferation, Apoptosis, and Transcription Factor Activities During the Promotion of Liver Carcinogenesis by Polychlorinated Biphenyls. *Toxicology and Applied Pharmacology* 179:172-184.

Timsit, Y.E,. & Negishi, M. 2007. CAR and PXR: The Xenobiotic-Sensing Receptors. *Steroids* 72: 231-246.

Tsai, P.C., Ko, Y.C., Huang, W., Liu, H.S., & Guo, Y.L. 2006. Increased Liver and Lupus Mortalities in 24-Year Follow-Up of the Taiwanese People Highly Exposed to Polychlorinated Biphenyls and Dibenzofurans. *Science of the Total Environment* 374:216-222.

Uehira, M., Uno, M., Kurner, T., et al. 1989. Development of Autoimmune Insulitis is Prevented in Eα but not in Aβ NOD Transgenic Mice. *Int. Immunol.* 1:209-213.

Uno, S., Imagawa, A., Sayama, K., et al. 2007. Macrophages and Dendritic Cells Infiltrating Islets With or Without Beta Cells Produce Tumour Necrosis Factor-α in Patients with Recent-Onset Type 1 Diabetes. *Diabetologia* 50:596-601.

Verner, M-A., McDougall, R., Glynn, A., et al. 2013. Is the Relationship Between Prenatal Exposure to PCB-153 and Decreased Birth Weight attributable to Pharmacokinetics? *Environ. Health Perspect.* 121:1219-1224.

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, DOI: 10.5772/22040. Available from: http://www.intechopen.com/books/type-1-diabetes-pathogenesis-genetics-and immunotherapy/the-role-of-t-cells-in-type-1-diabetes

Wang, Shu-Li., Yang, Chiu-Yueh., Tsai, Pei-Chien., Guo, & Yueliang Leon. 2008. Increased Risk of Diabetes and Polychlorinated Biphenyls and Dioxins: A 24-year Follow-Up Study of the Yucheng Cohort. *Diabetes Care* 31:1574-1579.

Weisglas-Kuperus, N., Patandin, S., Berbers, G.A.M., Sas, T. C.J., Mulder, P.G.H., Sauer, P.J.J., & Hooljkaas, H. 2000. Immunologic Effects of Background

Exposure to Polychlorinated Biphenyls and Dioxins in Dutch Preschool Children. *Environmental Health Perspectives* 108:1203-1207.

WHO. 2008. Training for the Health Sector-Persistent Organic Pollutants-Children's Health and the Environment. Retrieved From www.who.int/ceh Accessed on 01/20/2014.

Wicker, L.S., Todd, J.A., Prins, J., Podolin, P.L., Renjilian, R.J., & Peterson, L.B. 1994. Resistance Alleles at Two Non-Major Histocompatibility Complex-Linked Insulin-Dependent Diabetes Loci on Chromosome 3, *Idd3* and *Idd10*, Protect Nonobese Diabetic Mice from Diabetes. *J. Exp. Medicine* 180:1705-1813.

Wojtowicz, A., Ropstad, E., & Gregoraszczuk, E. 2001. Estrous Cycle-Dependent Changes in Steroid Secretion by Pig Ovarian Cells Exposed in vitro to Polychlorinated Biphenyl (PCB 153). *Endocrine Regulations* 35:223-228.

Zhang, B., Choi, J.J., Eum, S.Y., Daunert, S., & Toborek, M. 2013. TLR4 Signaling Is Involved in Brain Vascular Toxicity of PCB153 Bound to Nanoparticles. *PLoS ONE* 8(5) 1-11. doi:10.1371/journal.pone.0063159

Zhang, Q., Lu, M., Wang, C., Du, J., Zhou, P., & Zhao, M. 2014. Characterization of Estrogen Receptor α Activities in Polychlorinated Biphenyls by *in vitro* Dual Luciferase Reporter Gene Assay. *Environmental Pollution* 189:160-175.