Effects of gestational exposure to mercury on mitogen and antigen-specific immune responses

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ABSTRACT

Because *in utero* exposure to mercury has been shown to induce phenotypic changes in fetal immune cells that persist in adult offspring, we examined the effects of *in utero* exposure to mercuric chloride ($HgCl_2$) on the immune response to an antigen, DNP-KLH. Pregnant BALB/c dams received either plain tap water or water containing 10ppm $HgCl_2$ *ad libitum* throughout gestation, and were switched to plain water after parturition. Adult offspring were immunized with 100μg DNP-KLH, and six weeks later, splenocytes were analyzed for immune phenotype and function. $HgCl_2$ exposure resulted in alterations in splenocyte phenotype in response to DNP-KLH in male and female mice and increased proliferation of splenic lymphocytes to ConA or LPS; in female mice, there was a specific increase in the proliferative response to LPS. $HgCl_2$ exposure did not affect IL-2 production by splenocytes in response to DNP-KLH. There was no effect of $HgCl_2$ exposure on IFN-γ or IL-4 production; however, the production of IFN-γ or IL-4 in response to DNP-KLH was greater in mercury-exposed male versus female mice. IL-10 production by splenocytes in response to ConA was greater in mercury-exposed male versus female mice. After cells were cultured in media alone, cells from male mice produced greater levels of DNP-KLH-specific IgG as a result of $HgCl_2$ exposure during gestation. $HgCl_2$ exposure did not significantly affect the production of the DNP-KLH-specific immunoglobulins in response to DNP-KLH. Taken together, these data suggest that *in utero* exposure to $HgCl_2$ may result in long-term gender-specific alterations of immune system responses.

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INTRODUCTION

Exposure to the heavy metal mercury is known to be detrimental to health. Such effects on health have been observed in previous research as alterations of the nervous system and alterations in renal function. Mercury is also known to be transferred from a mother to her fetus during gestation, which has implications for fetal health and development. Of great concern are the recent research findings that *in utero* exposure to mercury has effects on the immune system, and these effects last into adulthood. In this study, we wanted to determine the alterations, resulting from gestational exposure to mercury, in the function of the immune response to a specific antigen, DNP-KLH. We also wanted to analyze possible gender-specific effects of gestational mercury exposure as a result of mercury's potential role as an endocrine disruptor.

LITERATURE REVIEW

People may be exposed to heavy metals in a variety of ways. The contamination of soil samples with the heavy metals zinc, cadmium, and lead at a now defunct paint factory in the city of Changchun, China (13) and the high concentrations of mercury, arsenic, lead, and zinc in the industrial area of Estarreja, Portugal (14) are only two examples of high levels of heavy metal contamination of the environment. There are also routes of exposure to lower levels of heavy metals. Even the United States, which has regulations for heavy metal contamination, allows Maximum Contaminant Levels (MCLs) in drinking water for heavy metals such as cadmium and inorganic mercury (15).

The exposure to environmental contaminants, such as mercury, then poses the risk of continued health problems around the world (16, 17).

Mercury is present in the environment in three general forms: metallic mercury, also known as elemental mercury, inorganic mercury, and organic mercury. The inorganic mercury species, such as mercuric chloride, are formed when mercury combines with the molecules sulfur, oxygen, or chlorine, while forms of organic mercury, such as methylmercury, are produced when mercury is combined with carbon. The common natural forms of mercury include mercuric chloride, methylmercury, mercuric sulfide, and metallic mercury. Although mercury is normally present in the environment due to natural deposits and volcanic activity, water, soil, and air are also contaminated with mercury due to waste disposal, the burning of coal and wastes, and the output of manufacturing plants. As a result, hazardous waste sites have been found to have higher levels of mercury than other areas (4).

Common routes of human exposure to mercury are contaminated food, contaminated work environments, dental amalgam fillings, and cultural practices. In contaminated fresh and salt water, methylmercury can accumulate in the tissues of fish and other marine animals that may later be consumed by humans. To prevent high levels of mercury consumption, the Food and Drug Administration (FDA) has set the maximum allowable level of mercury in seafood at 1 part per million (ppm) and estimates the daily exposure to mercury from food at 50ng per kilogram of body weight. The Environmental Protection Agency (EPA) has set the oral reference dose (Rfd) for methylmercury, which

is the maximum amount of methylmercury that will not result in significant risk of toxicity if absorbed daily, at 0.1µg/kg/body wt./day (34). Certain work environments, such as those involving mining or manufacturing, may expose the workers to mercury. The Occupational Safety and Health Administration (OSHA) enforces maximum levels of 0.1mg/m^3 organic mercury and 0.05mg/m^3 metallic mercury vapor in the air at the workplace. Metallic mercury exposure has also occurred from the use of dental amalgam fillings because mercury comprises approximately 50% of the amalgam. Due to the slow breakdown of the amalgam as a result of chewing and tooth damage, mercury is slowly released from the amalgam (4). Of concern, the amalgam fillings not only expose a pregnant woman to mercury, but her unborn children are exposed as well. A study found a correlation between the number of dental amalgam fillings in the mother and the concentration of mercury in the placenta (5), and the concentration of inorganic mercury in breast milk (9). Dental amalgam is still used as a material for fillings, although it is now more commonly used for teeth located in the back of the mouth (33). Finally, although exposure to mercury is often accidental, some humans purposely expose themselves to mercury. During some cultural and religious practices found in areas of Latin America and the Caribbean, metallic mercury is used as part of certain religious rituals and herbal remedies (4).

Mercury may enter the body by several different means. It can enter the body by the swallowing of contaminated food or water, by the inhalation of mercury from the air, or by the absorption of mercury through the skin. The route of entry and the form of mercury determine the levels of mercury found in the body by affecting the absorption

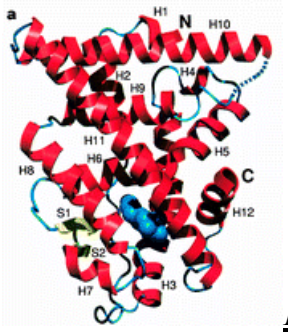
rate. When metallic mercury was swallowed, less than 0.01% was found to be absorbed into the body, but when inhaled, approximately 80% of the metallic mercury was absorbed (4). Inorganic mercury is not easily absorbed if inhaled, and less than 10% is absorbed when swallowed (4, 23), although that level of absorption can be as high as 40% (4). Methylmercury, a form of organic mercury, has a 95% absorption rate if swallowed and also has a high rate of absorption if inhaled; other forms of organic mercury are quickly absorbed when in contact with skin (4).

As mentioned previously, after mercury enters the body, it can cross the maternal-fetal barrier leading to exposure of the fetus to mercury, which makes mercury exposure especially dangerous for pregnant women. *Schober et al.* surveyed U.S. women of childbearing age (ages 16-49) and found that 7.8% of the women had levels of mercury in the blood above the Rfd of 5.8μg/L (35), and another study found that 1-3% of U.S. women of childbearing age (ages 15-44) eat amounts of fish that are great enough to put them at risk for methylmercury exposure (34). Both studies indicate the risk of mercury exposure for women of childbearing age and have implications for gestational exposure to mercury in humans. In a study that involved pregnant women in Sweden, inorganic mercury was found to be in the placenta at concentrations four times higher than the concentration of mercury in maternal blood. Inorganic mercury was also found in umbilical cord blood at a concentration similar to the concentration in maternal blood (5). Mercury has also been found to enter the breast milk of women who have been exposed to mercury. In lactating women, a significant correlation was found between the levels of mercury in the blood and the levels of mercury in milk, which has implications for

breastfeeding mothers and their children (9). There are persistent effects on the children as a result of mercury exposure. As a result of gestational exposure to methylmercury, due partially to the consumption of fish and whale by the mothers, children were found to have decreased Neurologic Optimality Scores that correlated with an increased mercury concentration in the cord blood (7). This suggests that a possible health risk after gestational exposure to mercury is impaired neurological function (7). These effects are long-term; altered neurobehavioral function was found fourteen years after prenatal exposure to methylmercury in children living in the Faroe Islands. Further, higher levels of mercury exposure during gestation were found to be associated with decreased scores in neurobehavioral tests, such as finger tapping (10).

Other studies have also documented effects of exposure to mercury on other organs and organ systems of the body. Mercury exposure has been shown to affect kidney function. An exposure-response relation between mercury levels in the environment and the risk of death from kidney disease was found in the area of Runcom, England (8). The cardiovascular system has also been shown to be affected by mercury exposure. *Guallar et al*. found an increased risk of myocardial infarction with greater measured levels of exposure (18), and *Oliveira et al*. observed alterations in contractile activity of cardiac muscle from a rat model after exposure to mercuric chloride (19). These studies show that the health implications of mercury exposure are persistent and very diverse.

Other studies have examined the mechanisms by which mercury affects target organs and systems. Mercury has been shown to act as an endocrine disruptor in human breast cells by having estrogen-like activity (20, 21). Exposure to mercury was found to significantly stimulate the growth of cells in comparison to cells in media without estrogen, but this observed proliferation was prevented by the addition of antiestrogen. This result suggested an interaction between mercury and the estrogen receptor-α protein (ER_α) (Fig.1). Both endogenously and exogenously expressed estrogen receptors were activated by mercury, and evidence was found to suggest that mercury competed with estradiol for binding to ER_α (20). Mercury's possible role as an endocrine disruptor has implications for the immune system because estrogens and ER_α have been found to play a role in the normal development of the thymus (27, 28). Further, estrogen receptors have been shown to be involved in the function of immune cells, and interaction of ER_α on these cells with estrogen can lead to their up-regulation (32).



<u>Fig. 1</u>: The estrogen receptor-α protein (ER_α) shown in a ribbon model with a view of the ligand-binding domain (LBD). E_2 is shown as the blue space-filling model bound to ER_α (22). Mercury is thought to interact with specific amino acids of the receptor, especially cysteines, histidines, glutamic acids, and aspartic acids (20).

After mercury exposure, in studies on the distribution of mercury in the body, mercury was found to accumulate in areas such as the lymph nodes, thymus, and spleen

(24, 25). After the exposure of human T lymphocytes to mercuric chloride, *Guo et al*. observed the activation of apoptotic signaling pathways within the cells (29); however, data from humans has been found to be inconsistent and limited. For example, one study found no effects of mercury in the cord blood in humans on the expression of activated surface markers (CD4+, CD8+, and CD3+) (36), but a study by *Belles-Isles et al.* found a negative association between the concentration of mercury in the cord blood and the number of naïve helper T cells (37). In a mouse model, persistent effects on the immune system have been observed after gestational exposure to mercury (1, 6, 11). *Pilones et al*. found that gestational exposure to mercury in a mouse model resulted in altered lymphocyte phenotypes in the thymuses of gestation day (g.d.) 16 fetuses. In the thymus, an increased presence of double negative (CD4-CD8-) lymphocytes was observed (6). *Silva et al*. found decreased IFN-γ production as a result of gestational mercury exposure and also observed gender differences in the effects. In female mice, mercury was found to have an inhibitory effect on cytokine production; whereas, in male mice, mercury was observed to have a stimulatory impact on cytokine production (11). Another study found that *in utero* mercuric chloride exposure resulted in alterations of the immune system that persisted in 10-week-old DBF_1 mice (1). Such alterations included increased proliferative responses of splenocytes to the mitogens Concanavalin-A (ConA) and Lipopolysaccharide (LPS) and increases in the production of the cytokines IFN-γ and IL-4 in response to ConA stimulation. Gender-specific effects were also found, with female mice having more phenotypic changes in T lymphocytes than male mice (1). Thus, it is possible that the persistent effects of mercury on the immune system could pose a

possible health risk associated with an alteration of the immune response to infectious agents and of an induction of aberrant or allergic immune responses.

AIMS AND SIGNIFICANCE

Following from the previous research, this study was designed to test the hypothesis that *in utero* exposure to mercuric chloride could lead to alterations in the adult immune response to antigen. In order to test this hypothesis, (BALB/c x DBA/1)F_1 (DBF_1) mice (1,6) that had been exposed to mercury at 10mg/L $HgCl_2$ in the drinking water for the length of gestation were used (1). Estimating a daily water intake averaging 2.5mL per dam and a 10% absorbance level of mercury across the gastrointestinal tract, the daily mercuric chloride exposure was approximated at 208µg/kg body weight (1), which is below previously observed extremely fetotoxic doses (26). After the F_1 progeny reached adulthood, the mice received a single intra peritoneal (i.p.) injection of a specific antigen, 2,4-dinitrophenyl keyhole limpet hemocyanin (DNP-KLH) (Fig. 2). Six weeks later, the spleens were harvested for analysis.

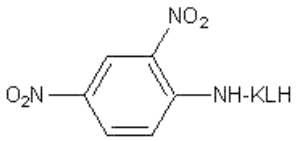


***Fig. 2*: The molecular structure of the antigen, DNP-KLH (12)**.

The modulations of the immune system challenged with DNP-KLH after *in utero* mercuric chloride exposure were analyzed by looking at splenic cellularity, flow

cytometric analysis of the splenic cell phenotypes, the proliferative responses of the splenic lymphocytes to mitogen and DNP-KLH, T_H1 and T_H2 cytokine production by splenic lymphocytes, and the production of the DNP-KLH-specific immunoglobulins: IgM, IgG (IgG1, IgG2a, and IgG2b), and IgE.

In conclusion, it is well documented that humans are regularly exposed to low levels of mercury in the environment. Exposure to mercury is often the result of the consumption of food, especially fish, containing mercury. The exposure of a pregnant woman to mercury is known to result in the exposure of the fetus to mercury, which has health implications for the fetus. It has also been shown that the immune system is altered by mercury exposure, and these effects are persistent after *in utero* exposure to mercury. The work reported in this thesis is highly relevant to human health as it will further characterize the persistent effects of gestational exposure to mercuric chloride on the immune system, in particular, the possible effects of mercury exposure on the immune response to an antigen. This has potential health implications for the ability of the immune system to respond to a pathogen and to respond to immunization after gestational exposure to mercury. More information about the health risks of *in utero* mercury exposure on the function of the immune system should be gained from the results of this study.

MATERIALS AND METHODS

Mice

Eight-week old BALB/c female and DBA/1 male mice were acquired from The Jackson Labs (Bar Harbor, ME) and set up into harems of three BALB/c females and one DBA/1 male (1). After overnight breeding, pregnancies were detected by the presence of a vaginal plug, and that day was designated as day 0 of gestation. Pregnant females were placed into individual polycarbonate cages and randomly designated to the control group or the mercury-exposed group. Mercuric chloride ($HgCl_2$) (Sigma, St. Louis, MO) was prepared in endotoxin- free tap water to a concentration of 10mg/L (10 ppm). Beginning at gestation day (g.d.) 0, the eight pregnant females in the mercury-exposure group were given drinking water containing 10ppm $HgCl_2$ *ad libitum,* and the four pregnant females in the control group were given regular tap water *ad libitum*. At parturition, the dams in the mercury-exposed group were supplied with regular drinking water *ad libitum*. The DBF_1 offspring were weaned at day 21, randomized within exposure group, and then housed by sex. A total of six DBF_1 females and nine DBF_1 males that had been exposed to mercuric chloride *in utero*, and three DBF_1 female and three DBF_1 male mice that had not been exposed to mercuric chloride *in utero* were used for this study. No mortalities were noted during the course of this experiment. The mice were handled in accordance with the specifications of the Institutional Animal Care and Use Committee (IACUC) of Cornell University.

Immunization with DNP-KLH

At approximately 38 weeks of age, all of the mice received an intraperitoneal (i.p.) injection of 100μg of 2,4-dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH) (Calbiochem, San Diego, CA). Six weeks after the injections, the mice were euthanized, and the splenocytes were tested for their immune phenotypes and function.

Harvest of splenic tissue and preparation of splenocyte suspensions

After the mice were euthanized by CO_2 asphyxiation, the spleens were removed aseptically, and placed in 5mL of RPMI 1640 media (BioWhittaker, Walkersville, MD, USA). Frosted glass slides were used to emacerate the spleens into a single cell suspension, and the erythrocytes lysed by treatment with 5mL Tris-ammonium chloride (TAC) (155mM NH_4Cl/34mM Tris pH 7.2) (Sigma) for 5 minutes at room temperature. After the addition of 5mL of RPMI 1640 media to the tubes, the tubes were centrifuged at 229 x g (1100 rpm) for 5 minutes to pellet the cells. The supernatant in each tube was aspirated off, and the cells were re-suspended in 1mL of RPMI supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, L-glutamine and 1% non-essential amino acids (Sigma) (complete RPMI). The cells were then counted by using trypan blue exclusion. The cells were also used to prepare cell suspensions for flow cytometric analysis, for proliferations assays, for cytokine assays, and for DNP-KLH-specific immunoglobulin assays.

Flow cytometric analysis of splenocyte phenotype

Equivalent numbers of cells from individual mice in each exposure group were pooled, based on sex, to a final concentration of 1 x 10^6 cells/mL in 5mL total volume complete RPMI. The cells were then cultured with DNP-KLH (400µg/mL), and incubated for 72 hours at 37°C in an atmosphere of 5% CO_2. The cells were then pelleted and prepared for flow cytometry to analyze the phenotypes of the lymphocytes in the spleen. Total splenocytes were dispensed at a level of $1x10^5$ cells per tube, and were washed with 500µL PBS containing 0.5% bovine serum albumin (BSA) and 0.1% NaN_3 (PBS-B-Azide Wash buffer) (Sigma), and then spun down at 208 x g (1000 rpm) for five minutes. Supernatants were removed, and the cells were then re-suspended in wash buffer. The cells were stained with optimum dilutions of monoclonal antibodies: PE-conjugated anti-mouse CD8, FITC-conjugated anti-mouse CD4, PerCP-conjugated anti-mouse CD25, APC-conjugated anti-mouse CD44, PE-Cy7-conjugated anti-mouse CD3, FITC-conjugated anti-mouse GITTR, PE-conjugated anti-mouse NK1.1, PerCP-conjugated anti-mouse CD11B, and APC-conjugated anti-mouse CD45R (B220) (BD Pharmingen, San Diego, CA). The cells were incubated on ice in the dark for 20 minutes and then washed with 500µL PBS containing 0.5% BSA and 0.1% NaN_3. In order to fix the cells, 200µL of 1% paraformaldehyde (Sigma) in PBS was added to the tubes, and the tubes were kept at 4°C until the flow cytometric analysis was completed. The analysis of the single replicates was completed using a FACS LSR II Flow Cytometer (Becton Dickinson, Mountain View, CA), and the data analyzed using FlowJo (Tree Star, Inc., Ashland, OR) and the WinMDI analysis program (Scripps Research Institute FACS Core

Facility (http://facs.scripps.edu/software.html)). The specificity of the antibodies used and a description of the antigens are shown in Table 1.

Table 1: Stains for Flow Cytometry

Antigen	Protein expression	Stain type
CD4	Protein expressed on T cells restricted to interaction with MHC Class II molecules	FITC
CD8	Protein expressed on T cells restricted to interaction with MHC Class I molecules	PE
CD25	α-chain of IL-2 receptor	PerCP
CD44	Adhesion protein found on immature B and T cells	APC
CD3	T cell receptor expressed on mature T cells	PE-Cy7
GITTR	Expressed on T regulatory cells	FITC
NK1.1	Expressed on surface of natural killer cells	PE
CD11B	Expressed on monocyte/ macrophage cells	PerCP
CD45R (B220)	Expressed on all B cells and on a small number of dendritic cells	APC

Proliferation assay

Lymphocytes obtained from the spleen were analyzed for their *in vitro* proliferative ability using the Promega CellTiter96® Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI). Cells from individual mice at a final concentration of $5x10^5$ cells/well were cultured in complete RPMI media in 96-well Microtest III Tissue Culture Plates (Becton Dickinson Labware, Franklin Lakes, NJ). Cells (50µL/well) were cultured in triplicate in media alone, (50µL), with ConA, at 50µL/well (40µg/mL) (Sigma), with 50µL LPS (40µg/mL), or with 50µL DNP-KLH (400µg/mL). Total culture volume was 100µL/well. The cells were incubated at 37°C in an atmosphere of 5% CO_2 for 72 hours. The proliferative responses were then measured by the addition of 15µL MTT dye solution, followed by incubation at 37°C for 4 hours, and then 50µL of stop solution was added to each well. After an additional 1-4 hours incubation, the absorbance was read at 492nm using a Tecan Genios Fluorescence,

Absorbance, and Luminescence Reader (MTX Lab Systems, Inc., Vienna, VA). Data were analyzed for significance using an analysis of variance (ANOVA) (2-factor) in SAS 9.1 software (SAS Institute, Inc., Cary, North Carolina). Two-sample Student's *t*–tests with unequal variances in the Microsoft Excel software were also used (Microsoft Corp., Seattle, WA.).

ELISA for in vitro cytokine production

Enzyme-linked immunosorbent assay (ELISA) was used to measure the levels of the cytokines IL-2, IL-4, IL-10, and IFN-γ in the supernatants of the cultured cells. Splenocytes from individual mice were diluted to a final concentration of $1x10^6$ cells/well in complete RPMI media to a total volume of 1mL in 24-well Cell Culture Cluster plates (Corning Incorporated, Corning, NY). Cells were cultured in media alone for unstimulated cells. In other cultures, the cells were stimulated with ConA (40µg/mL) at a final concentration of 2µg/mL or with DNP-KLH (400µg/mL) at a final concentration of 20µg/mL. The cultures were incubated at 37°C in an atmosphere of 5% CO_2 for three days, and the plates were then frozen at –70°C until the supernatant was assayed for cytokine production by ELISA as previously described (1). Briefly, 96-well NUNC-Immuno® Maxi-Sorp plates (Nalgene, NY) were coated with 50µL anti-mouse IL-2, IL-4, or IFN-γ (eBioscience, San Diego, CA) in 0.1M carbonate coating buffer, pH 9.5, or anti-mouse IL-10 (eBioscience) in 0.1M phosphate coating buffer, pH 6.5 overnight at 4°C. Anti-mouse IL-2 was diluted to a concentration of 2µg/mL, anti-mouse IL-4 was diluted to a concentration of 2µg/mL, anti-mouse IL-10 was diluted to a concentration of 0.5µg/mL, and anti-mouse IFN-γ was diluted to a concentration of 0.5µg/mL. For the

assay, the plates were washed two times with 150µL phosphate-buffered saline (PBS)/0.05% Tween (Tw) (Sigma), blocked with 50µL PBS/10% FBS (Sigma), pH 7 for one hour at room temperature, and then washed twice with 150µL PBS/0.05% Tw. Undiluted supernatant samples were dispensed into the wells at volumes of 50µL in duplicate. After overnight incubation at 4°C, the plates were washed three times with 150µL PBS/0.05% Tw, and then 50µL of biotinylated anti-mouse IL-2 (diluted 1:250 in PBS/10% FBS, pH 7), IL-4, IL-10, or IFN-γ (eBioscience) (diluted 1:500 in PBS/10% FBS, pH 7) was added to the wells. After an additional overnight incubation at 4°C, the plates were washed four times with 150µL PBS/0.05% Tw, and each well received 50µL streptavidin-horseradish peroxidase (SAV-HRP) (BD Biosciences Pharmingen, San Jose, CA) (diluted 1:1,000 in PBS/10% FBS). After 30 minutes at room temperature, the plates were washed five times with 150µL PBS/0.05% Tw, and 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma) was added at 50µL per well. Color development proceeded for 15-30 minutes at room temperature, and then the reaction was stopped by adding 50µL 1M H_3PO_4. Absorbance was read at 450nm using a Tecan Genios Fluorescence, Absorbance, and Luminescence Reader (MTX Lab Systems, Inc.). Significance in the data was determined by using SAS 9.1 software to calculate ANOVA (2-factor) (SAS Institute, Inc.). Microsoft Excel software (Microsoft Corp.) was also used to calculate the values for the two-sample Student's *t*-test with unequal variances.

ELISA for <u>in vitro</u> production of DNP-KLH-specific immunoglobulins

Direct binding ELISA was used to determine the levels of IgM, IgE, and IgG (IgG1, IgG2a, and IgG2b) specific for DNP-KLH produced by cultured splenocytes.

Splenocytes from individual mice were dispensed into the wells of 24-well Cell Culture Cluster plates (Corning) to a final concentration of $5x10^5$ cells/well in complete RPMI media. The cells were cultured in either media alone, with 50µL LPS (40µg/mL), or with 50µL DNP-KLH (400µg/mL) for a total volume of 200µL in each well, for 7 days at 37°C in an atmosphere of 5% CO_2. The supernatant was then frozen at –70°C until the assays were performed. Immulon 1B plates (Dynatech Laboratories, Chantilly, VA) were prepared for the assay by coating with 50µL/well of 5µg/mL DNP-KLH in carbonate coating buffer (0.1M carbonate coating buffer, pH 9.5), followed by incubation at 4°C overnight. For the assay, the plates were washed twice with 150µL PBS/0.05% Tw and blocked with 50µL PBS/10% FBS for one hour at room temperature. Then, the plates were washed two times with PBS/0.05% Tw. The supernatants, diluted 1:2 in PBS/0.05% Tw, were added in duplicate at 50µL per well, and the plates were incubated overnight at 4°C. The plates were then washed three times with PBS/0.05% Tw. Biotin-Rabbit Anti-Mouse IgM (diluted 1:4,000 in PBS/0.05% Tw) (Zymed, Carlsbad, CA), Biotin Conjugated Anti-Mouse IgE (diluted 1:5,000 in PBS/0.05% Tw) (eBioscience), or Biotin Anti-Mouse IgG made up of: Biotin-Rabbit Anti-Mouse IgG1 (diluted 1:2,000 in PBS/0.05% Tw), Biotin-Rabbit Anti-Mouse IgG2a (diluted 1:4,000 in PBS/0.05% Tw), and Rabbit Anti-Mouse IgG2b (diluted 1:2,000 in PBS/0.05% Tw) (Zymed) were added in volumes of 50µL per well, and the plates were incubated overnight at 4°C. The plates were then washed with 150µL of PBS/0.05% Tw four times, and 50µL SAV-HRP (diluted 1:1,000 in PBS/0.05% Tw) was added to the wells. After thirty minutes at room temperature, the plates were washed five times with PBS/0.05% Tw. TMB substrate (Sigma) was added to the wells at 50µL/well, and the plates were incubated for 15-30

minutes at room temperature to allow time for color development, then 50µL 1M H_3PO_4 was added to each well to stop the reaction. A Tecan Genios Fluorescence, Absorbance, and Luminescence Reader (MTX Lab Systems, Inc.) was used to read the absorbance values at 450nm. Data were reported as Optical Density (O.D.) 450 nm, and statistical significance of differences was analyzed by ANOVA (2-factor) in SAS 9.1 software (SAS Institute, Inc.). Two-sample Student's *t*-tests with unequal variances were completed in Microsoft Excel (Microsoft Corp.).

RESULTS

Effect of gestational mercury exposure on splenic cellularity

We analyzed whether gestational exposure to mercuric chloride altered splenic cellularity in forty-four-week old mice. There were no significant differences in the numbers of splenocytes in mercury-exposed male and female mice compared to unexposed control male and female mice (male, $6.14 \pm 2.69 \times 10^7$ cells for mercury vs. $2.21 \pm 1.90 \times 10^7$ cells for control, and female, $5.84 \pm 2.06 \times 10^7$ cells for mercury vs. $6.21 \pm 4.49 \times 10^7$ cells for control, ANOVA: p=0.1979) (Fig. 3, Appendix 2). There were also no significant differences in the splenic cellularity of mercury-exposed and unexposed control female mice compared to mercury-exposed and unexposed control male mice (ANOVA, p=0.1807) (Fig. 3, Appendix 2).

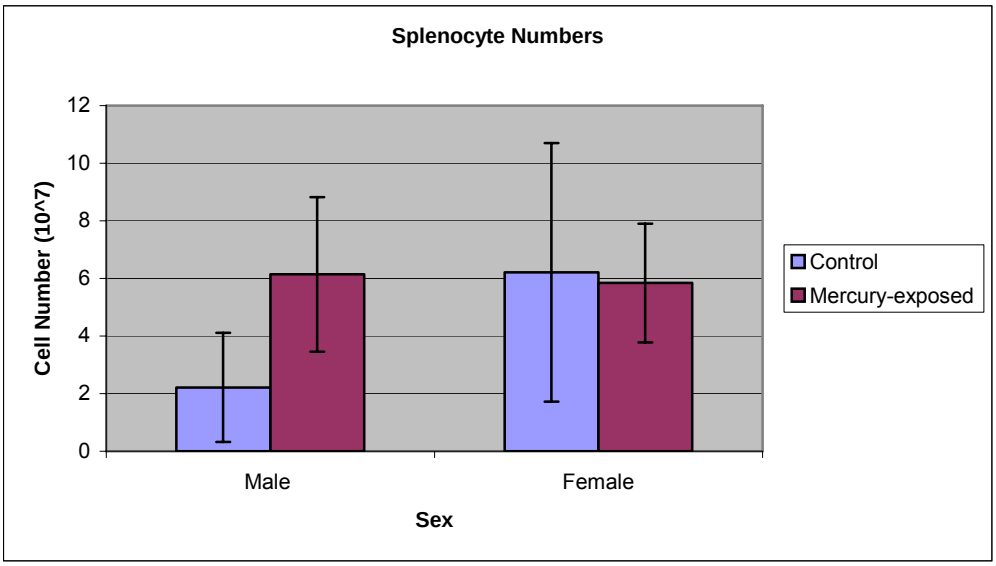


***Fig. 3*: The effect of mercuric chloride on spleen cell numbers. The data are shown as the mean cell number +/- S.D.**

Effect of gestational mercury exposure on splenic cell phenotypes after culture with DNP-KLH

We examined whether the phenotypes of the splenocytes in response to DNP-KLH in adults were modulated by *in utero* mercury exposure using flow cytometric analysis. Single replicates of cells pooled by exposure group and sex were analyzed. The percentage of cells expressing CD4+CD25+ was slightly greater in mercury-exposed male mice compared to unexposed control male mice, but total cell numbers co-expressing CD4+ and CD25+ were greater in mercury-exposed male mice compared to unexposed control male mice (Table 2). In contrast, mercury-exposed female mice showed both a decreased percentage and total number of CD4+CD25+ splenocytes compared to unexposed control female mice. Both mercury-exposed male and female mice showed slightly lower percentages of CD4+CD44+ splenocytes compared to gender-matched unexposed control mice, but although mercury-exposed female mice had lower numbers of CD4+CD44+ cells compared to unexposed control female mice, mercury-exposed male mice had greater numbers of CD4+CD44+ cells compared to unexposed control male mice (Table 2). In mercury-exposed female mice there was a slightly lower percentage of CD4+ cells and a slightly lower number of total CD4+ cells compared to unexposed control female mice, but in mercury-exposed male mice there was a greater percentage of CD4+ cells and a greater total number of CD4+ cells compared to unexposed control male mice (Table 2). Interestingly, the percentage and total number of B220+ cells in mercury-exposed female mice were less than the percentage and total number of B220+ cells in unexposed control female mice (Table 2, Fig. 4). Mercury-exposed male mice had a slightly greater percentage and slightly

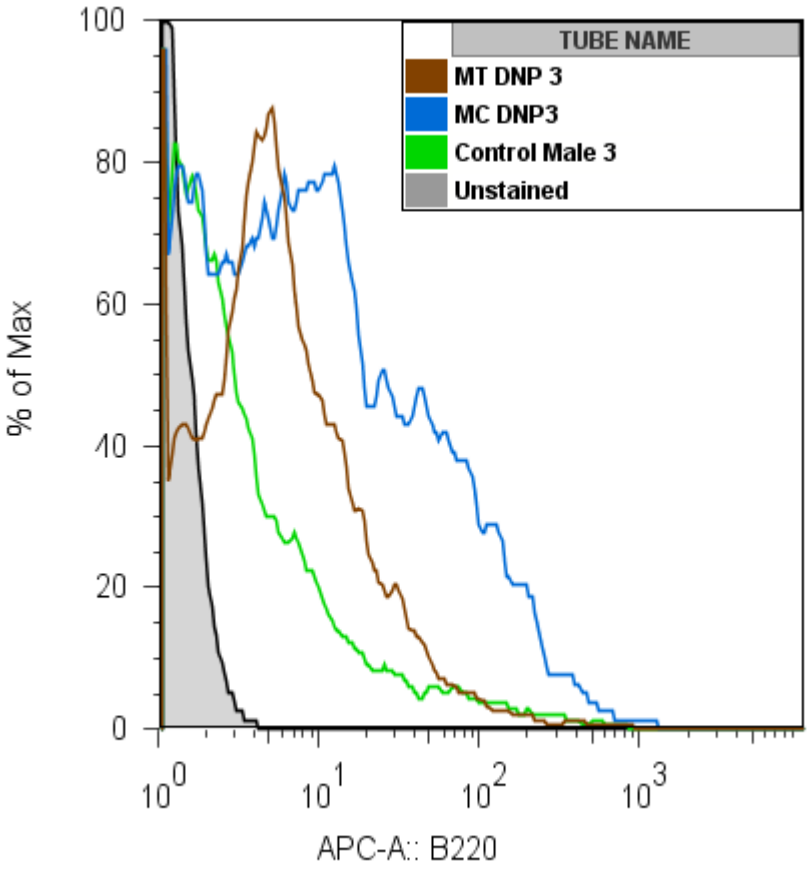
greater total cell number of NK1.1+ cells compared to unexposed control male mice; however, mercury-exposed female mice had a decreased percentage and decreased total cell number of NK1.1+ cells compared to unexposed control female mice (Table 2). Mercury-exposed female mice had a lower percentage and lower total number of GITTR+ cells in comparison to unexposed control female mice; however, mercury-exposed male mice had a greater percentage and total cell number of GITTR+ cells compared to unexposed control male mice (Table 2, Fig. 5).

Table 2: Percentages of splenocyte phenotype after gestational exposure to mercury.

Percentages	CD4+CD25+	CD4+CD44+	CD4+	B220+	NK1.1+	GITTR+
Unexposed control males	2.11	9.87	42.13	ND	0.13	0.62
Mercury-exposed males	2.25	8.99	49.29	ND	0.22	5.94
Unexposed control females	1.97	5.89	21.91	21.57	2.21	13.51
Mercury-exposed females	0.96	4.14	20.15	13.5	0.4	9.98

Absolute total cell numbers	CD4+CD25+	CD4+CD44+	CD4+	B220+	NK1.1+	GITTR+
Unexposed control males	4.67	21.8	93.25	ND	0.29	1.4
Mercury-exposed males	13.8	55.2	302.6	ND	1.4	36.5
Unexposed control females	12.2	36.6	136.1	134.0	13.7	83.94
Mercury-exposed females	5.6	24.2	117.7	78.8	2.0	58.3

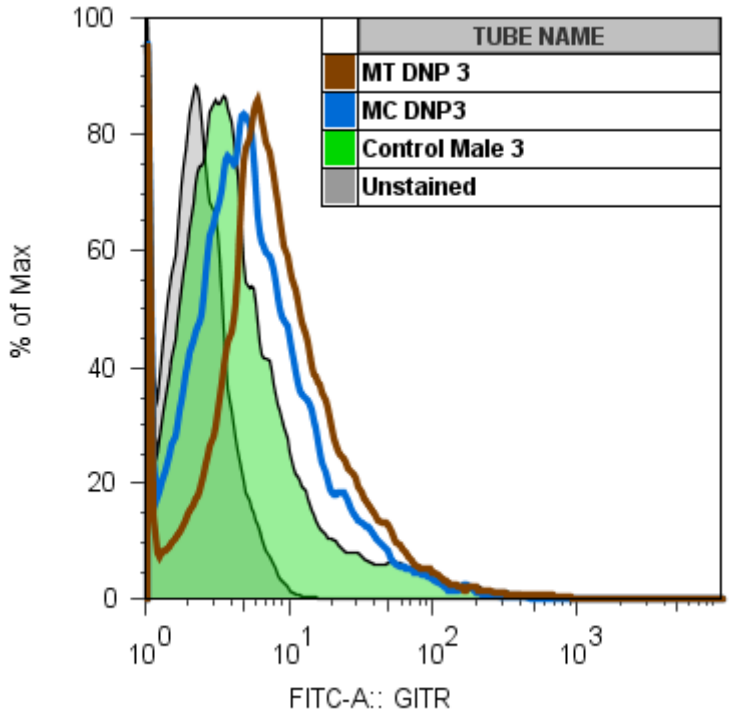
Mice were exposed <u>in utero</u> to 10ppm mercuric chloride and then immunized with DNP-KLH as adults at 38 weeks of age. Six weeks later, the phenotypes of the splenocytes in response to DNP-KLH were measured using flow cytometry. Absolute total cell numbers are expressed as cell number x 10^5 cells.



APC Lymphocyte gate

	MFI	# of cells
Unstained	0.03	2
Control	0.73	43
MC DNP 3	25.85	1324
MT DNP 3	3.45	186

***Fig. 4**: Effects of in utero exposure to $HgCl_2$ on the immune cell phenotype. Mice were exposed in utero to 10ppm $HgCl_2$ and then immunized with DNP-KLH as adults at 38 weeks of age. Six weeks later, the phenotypes of the splenocytes in response to DNP-KLH were measured using flow cytometry. Data shown are results of the APC stain for B220+ cells gated on lymphocytes. The tube MT DNP 3 is for mercury-exposed female mice, MC DNP 3 is for unexposed control female mice, and Control Male 3 is for unexposed control male mice. The chart shows the mean channel fluorescence (MFI) and the number of cells counted.*



FITC GITTR
Lymphocyte gate

	MFI	# of cells
Unstained	0.58	37
Control	4.2	247
MC DNP 3	10.02	464
MT DNP 3	15.45	834

***Fig. 5**: Effects of in utero exposure to $HgCl_2$ on the immune cell phenotype. Mice were exposed in utero to 10ppm $HgCl_2$ and then immunized with DNP-KLH as adults at 38 weeks of age. Six weeks later, the phenotypes of the splenocytes in response to DNP-KLH were measured using flow cytometry. Data shown are results of the FITC-A stain for GITTR+ cells gated on lymphocytes. The tube MT DNP 3 is for mercury-exposed female mice, MC DNP 3 is for unexposed control female mice, and Control Male 3 is for unexposed control male mice. The chart shows the mean channel fluorescence (MFI) and the number of cells counted.*

Effect of gestational mercury exposure on the proliferative response of splenocytes

The effects of *in utero* mercury exposure on the proliferative response of adult mouse splenocytes were measured in response to media alone, to the T lymphocyte mitogen ConA, to the B lymphocyte mitogen LPS, and, to evaluate secondary response to antigen, to DNP-KLH. There was an overall effect across culture conditions in which the proliferative response of splenocytes from mercury-exposed mice was greater than the proliferative response of splenocytes from unexposed control mice (0.46$\pm$0.23 for mercury vs. 0.28$\pm$0.04 for control, $p<0.01$) (Fig. 6, Appendix 3). Across culture conditions, splenocytes from mercury-exposed male and female mice showed greater proliferative responses compared to gender-matched unexposed control mice (male, 0.55$\pm$0.26 for mercury vs. 0.29$\pm$0.04 for control, $p<0.01$, and female, 0.33$\pm$0.08 for mercury vs. 0.27$\pm$0.04 for control, $p=0.01$) (Fig. 6, Appendix 3). When cells were cultured in media alone, cells from mercury- exposed mice did not show a significantly greater proliferative response than unexposed control mice (0.28$\pm$0.03 for control vs. 0.44$\pm$0.27 for mercury, ANOVA, $p=0.18$) (Fig. 6A, Appendix 3). After treatment with ConA, there was a significantly greater proliferative response of the splenocytes from mercury-exposed mice compared to unexposed control mice (0.49$\pm$0.19 for mercury vs. 0.29$\pm$0.06 for control, $p<0.01$) (Fig. 6B, Appendix 3). The splenocytes of mercury-exposed mice showed significantly greater proliferative responses after treatment with LPS compared to unexposed control mice (0.28$\pm$0.04 for control vs. 0.54$\pm$0.26 for mercury, $p<0.01$) (Fig. 6C, Appendix 3). There was also a specific significant difference in the proliferative response of splenocytes to LPS in female mercury-exposed mice compared to unexposed control female mice (0.40$\pm$0.05 for mercury vs. 0.29$\pm$0.05 for

control, p=0.05), which was not specifically observed in the proliferative response of splenocytes to LPS in mercury-exposed male mice compared to unexposed control male mice (0.63±0.31 for mercury vs. 0.27±0.02 for control, ANOVA, p=0.08) (Fig. 6C, Appendix 3). We did not find significant differences in the proliferative responses of splenocytes from mercury-exposed mice to DNP-KLH compared to cells from unexposed control mice (0.27±0.04 for control vs. 0.37±0.18 for mercury, ANOVA, p=0.21) (Fig. 6D, Appendix 3).

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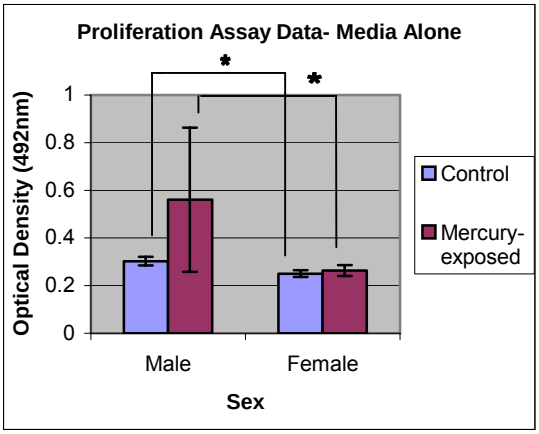

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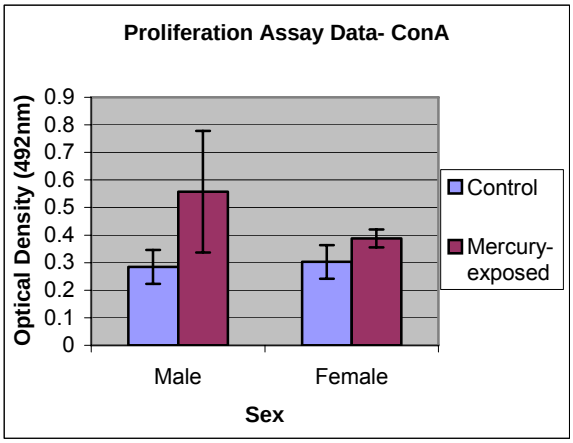

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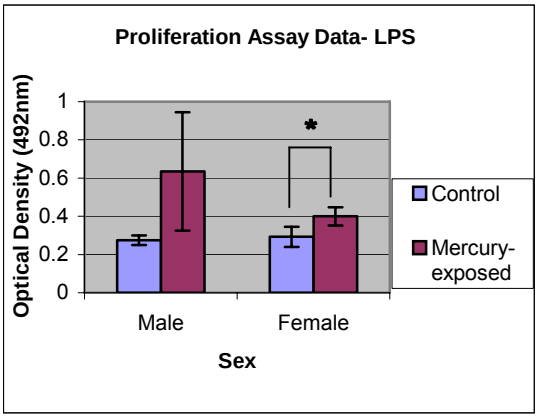

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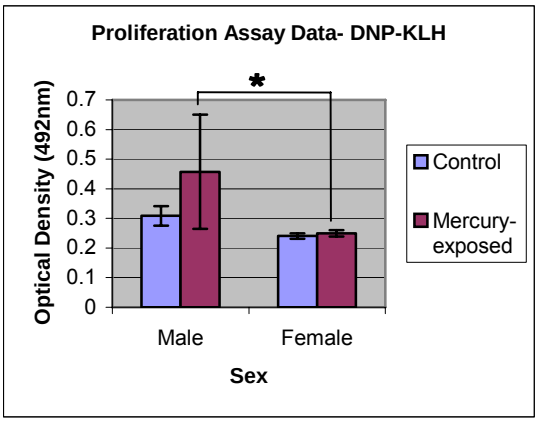

***Fig. 6**: Effects of in utero exposure to $HgCl_2$ on the immune response. Mice were exposed in utero to mercury and then immunized as adults at 38 weeks of age with DNP-KLH. Six weeks later, the proliferative responses of splenic lymphocytes in media alone (A), to the mitogens ConA (B) and LPS (C), and to DNP-KLH (D) were measured. Data were expressed as the mean O.D. at 492nm +/- S.D. Significant differences of p<0.05 using Student's t-test are indicated by an asterisk.*

Interestingly, we found significant gender differences in the proliferative responses of splenocytes. After culture in media alone, the splenocytes of unexposed control male mice showed a significantly greater proliferative response compared to unexposed control female mice (0.30±0.02 for male vs. 0.25±0.01 for female, p=0.02), and the splenocytes of mercury-exposed male mice showed a significantly greater

proliferative response compared to mercury-exposed female mice (0.56±0.30 for male vs. 0.26±0.02 for female, p=0.02) (Fig. 6A, Appendix 3). In response to DNP-KLH, the splenocytes of mercury-exposed male mice showed a greater proliferative response compared to mercury-exposed female mice (0.46±0.19 for male vs. 0.25±0.01 for female, p=0.01) (Fig. 6A, Appendix 3).

Effect of gestational mercury exposure on <u>in vitro</u> cytokine production

The effects of mercury exposure during gestation on the *in vitro* production of T_H1 and T_H2 cytokines in media alone and in response to ConA or DNP-KLH were measured by ELISA.

There were no significant differences observed across culture conditions in the production of IL-2 or IFN-γ in mercury-exposed mice compared to unexposed control mice (IL-2, 1.24±0.81 for mercury vs. 1.14±0.66 for control, p=0.60, and IFN- γ, 2.36±0.64 for mercury vs. 2.04±0.68 for control, p=0.10) (Fig. 7,8, Appendix 4A, 4B). No significant differences were measured between mercury-exposed male and female mice and gender-matched unexposed control mice in the production of IL-2 after cells were cultured in media alone (male, 0.78±0.06 for mercury vs. 1.18±0.39 for control, p=0.26, and female, 0.77±0.19 for mercury vs. 0.73±0.03 for control, ANOVA, p=0.70) (Fig. 7,8, Appendix 4A, 4B). Similarly, no significant differences were observed in the production of IFN-γ by cells from mercury-exposed mice compared to cells from unexposed control mice after cells were cultured in media alone (2.27±0.65 for mercury vs. 2.01±0.72 for control, ANOVA, p=0.49) (Fig. 7,8, Appendix 4A, 4B). In response to treatment with ConA, there was no difference in the production of IL-2 in cells from

mercury-exposed mice compared to cells from unexposed control mice (2.12±0.89 for mercury vs. 1.49±1.05 for control, ANOVA, p=0.14) (Fig. 7, Appendix 4A). We found no significant differences in the production of IFN-γ by cells from mercury-exposed mice in response to ConA compared to unexposed control mice (2.47±0.46 for mercury vs. 2.23±0.76 for control, ANOVA, p=0.45) (Fig. 8, Appendix 4B). Finally, there were no significant differences in the production of either IL-2 or IFN-γ in response to DNP-KLH in mercury-exposed mice compared to unexposed control mice (IL-2, 0.83±0.22 for mercury vs. 0.97±0.21 for control, ANOVA, p=0.23, and IFN-γ, 2.35±0.78 for mercury vs. 1.88±0.64 for control, ANOVA, p=0.21) (Fig. 7,8, Appendix 4A, 4B).

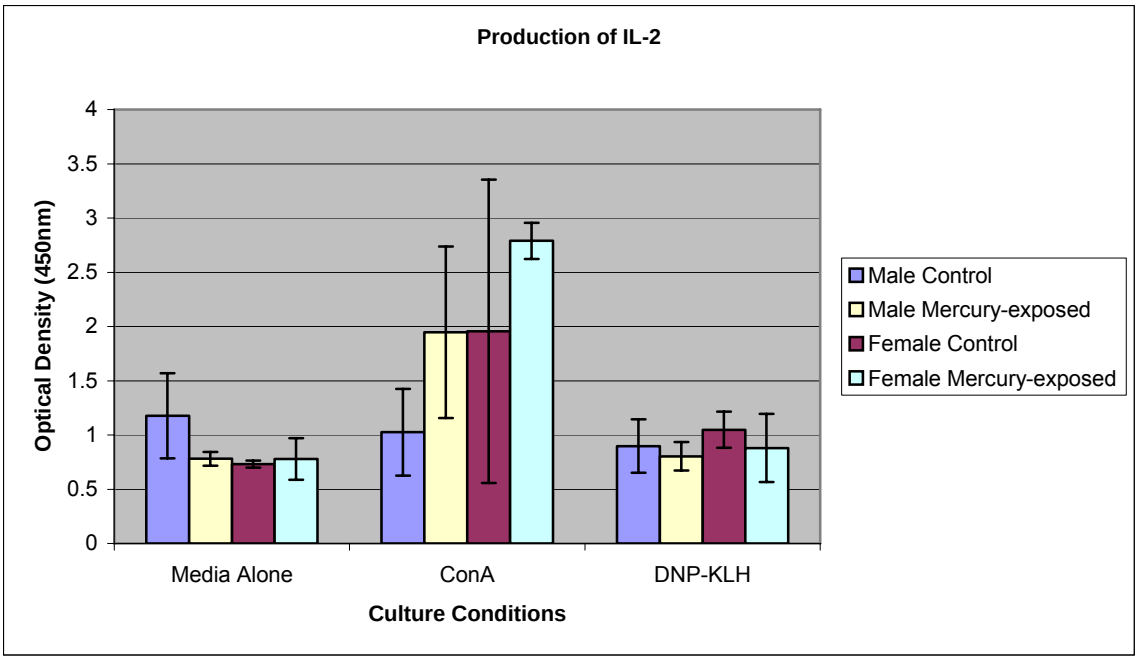

Fig. 7: The effect of in utero mercury exposure on the production of IL-2 by adult mice. Mice were exposed in utero to mercury and then injected as adults with DNP-KLH. Six weeks later, the splenic lymphocytes were harvested and the production of IL-2 in media alone or in response to treatment with ConA or DNP-KLH was measured. The data are shown as the mean optical density at 450nm +/- S.D.

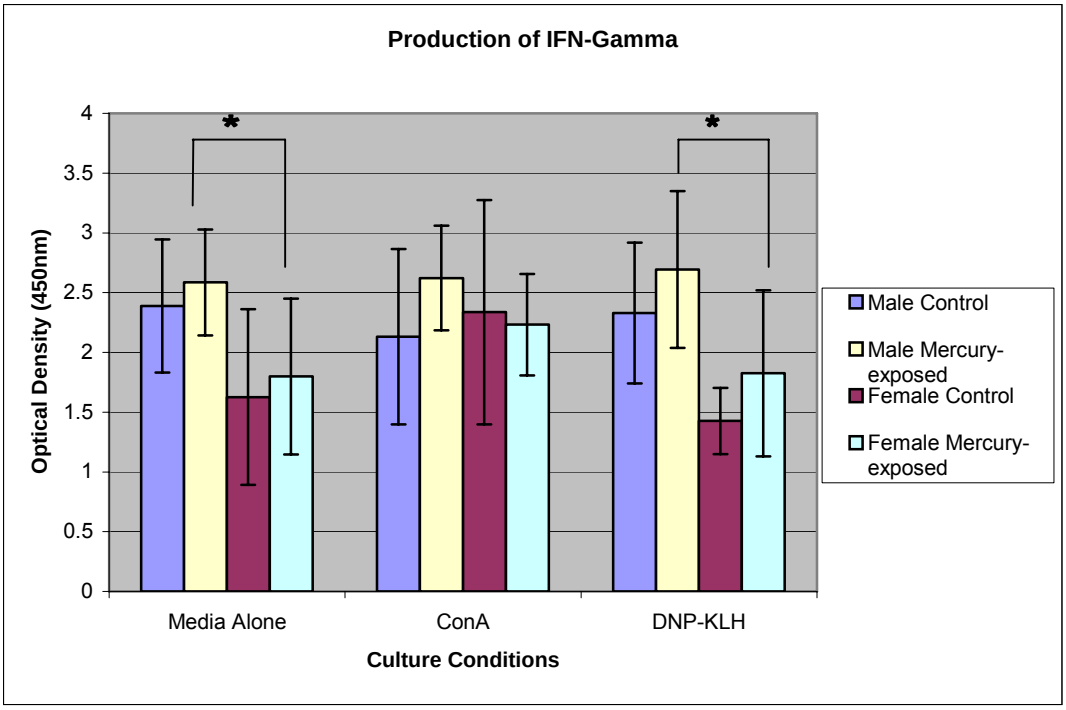

Fig. 8*: The effect of *in utero* mercury exposure on the production of IFN-γ by adult mice. Mice were exposed *in utero* to mercury and then injected as adults with DNP-KLH. Six weeks later, the splenic lymphocytes were harvested and the production of IFN-γ in media alone or in response to treatment with ConA or DNP-KLH was measured. The data are shown as the mean optical density at 450nm +/- S.D. Significant differences of $p<0.05$ using Student's t-test are indicated by an asterisk.

We noted that there were significant gender differences in the production of IFN-γ. Across culture conditions, there was greater production of IFN- γ by cells from mercury-exposed male mice compared to mercury-exposed female mice (2.63±0.50 for male vs. 1.95±0.60 for female, $p<0.01$); however, there was no difference observed in the production of IFN- γ by cells from unexposed control male mice compared to unexposed control female mice (2.28±0.56 for male vs. 1.80±0.74 for female, ANOVA, $p=0.14$) (Fig. 8, Appendix, 4B). Cells from mercury-exposed male mice cultured in media alone produced greater levels of IFN-γ compared to mercury-exposed female mice (2.59±0.44 for male vs. 1.80±0.65 for female, $p=0.03$) (Fig. 8, Appendix 4B). Antigen-specific

responses were greater in male mice; greater levels of IFN-γ were found in response to DNP-KLH in mercury-exposed male mice compared to mercury-exposed female mice (2.69±0.66 for male vs. 1.83±0.70 for female, p=0.03) (Fig. 8, Appendix 4B). Thus, as a result of gestational mercury exposure, we have found gender-specific effects in the production of IFN- γ.

The effects of gestational mercury exposure on the production of the T_H2 cytokines IL-4 and IL-10 by cells cultured in media alone or in response to treatment with ConA or DNP-KLH were also measured. There were no significant differences across all culture conditions in the production of IL-4 or IL-10 in mercury-exposed mice compared to unexposed control mice (IL-4, 1.81±0.58 for mercury vs. 1.73±0.59 for control, ANOVA, p=0.72, and IL-10, 2.13±0.38 for mercury vs. 2.07±0.51 for control, ANOVA, p=0.83) (Fig. 9,10, Appendix 4C, 4D). No significant differences were observed in the production of IL-4 or IL-10 after culture in media alone by cells from mercury-exposed mice compared to unexposed control mice (IL-4, 1.60±0.48 for mercury vs. 1.59±0.66 for control, ANOVA, p=0.81,and IL-10, 2.10±0.38 for mercury vs. 2.07±0.55 for control, ANOVA, p=0.94) (Fig. 9,10, Appendix 4C, 4D). There were no differences observed in the production of IL-4 in response to ConA by cells from mercury-exposed mice compared to unexposed control mice (2.30±0.47 for mercury vs. 2.08±0.51 for control, ANOVA, p=0.38) (Fig. 9, Appendix 4C). Similarly, in response to ConA, there was no difference in IL-10 production by cells from mercury-exposed mice compared to unexposed control mice (2.20±0.43 for mercury vs. 1.53±0.60 for control, ANOVA, p=0.51) (Fig. 10, Appendix 4D). In response to DNP-KLH, there were no

significant differences in the production of either IL-4 or IL-10 by cells from mercury-exposed mice compared to unexposed control mice (IL-4, 1.53±0.46 for mercury vs. 1.53±0.50 for control, ANOVA, p=0.74, and IL-10, 2.09±0.35 for mercury vs. 2.11±0.47 for control, ANOVA, p=0.71) (Fig. 9,10, Appendix 4C, 4D).

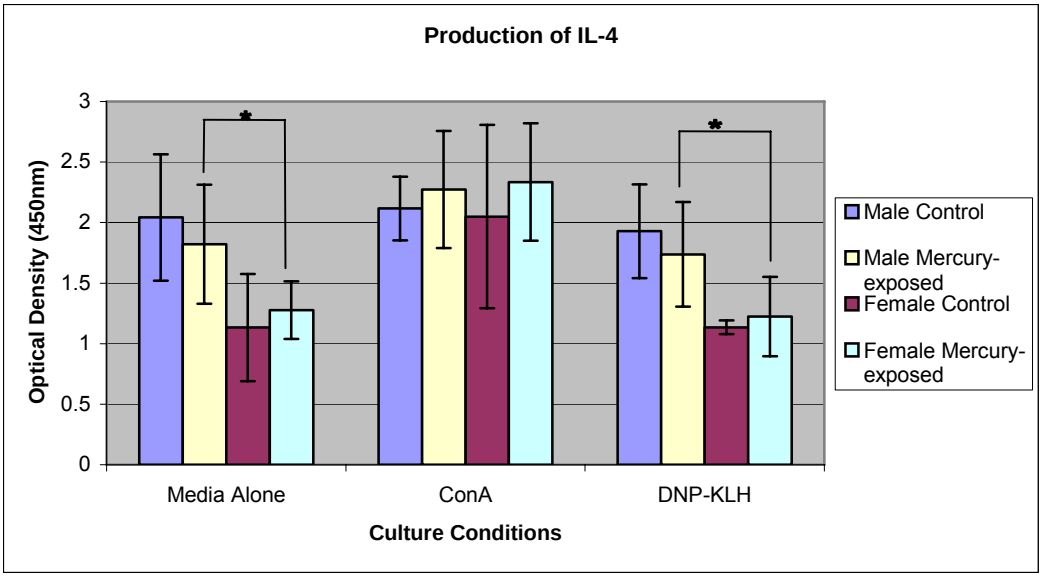

Fig. 9*: The effect of *in utero* mercury exposure on the production of IL-4. Mice were exposed *in utero* to mercury and then injected as adults with DNP-KLH. Six weeks later, the splenic lymphocytes were harvested and the production of IL-4 in media alone or in response to treatment with ConA or DNP-KLH was measured. The data are shown as the mean optical density at 450nm +/- S.D. Significant differences of $p<0.05$ using Student's t-test are indicated by an asterisk.

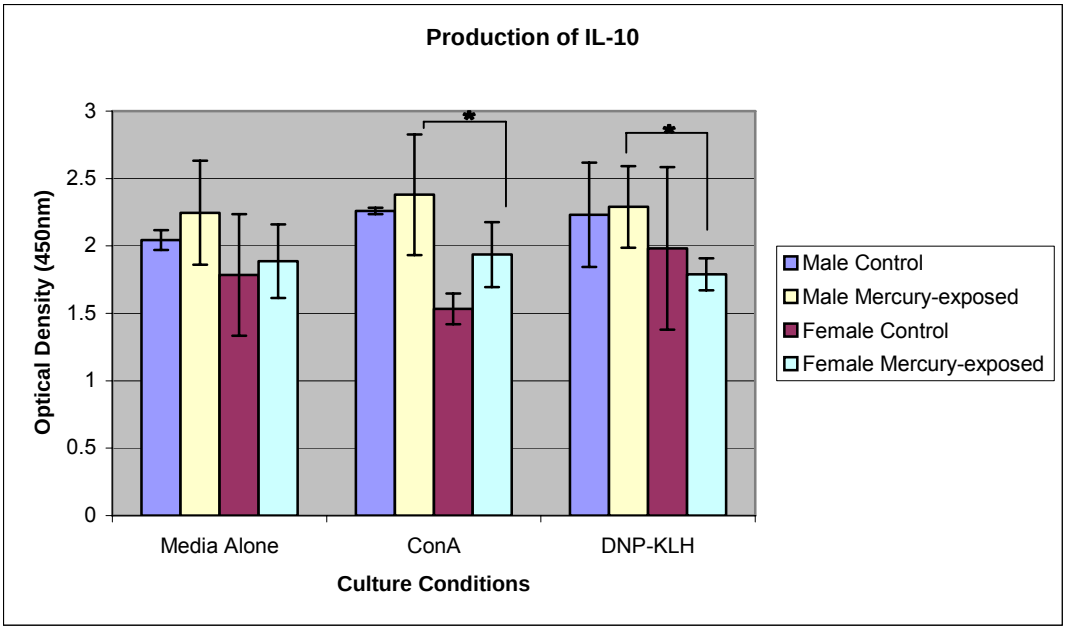

***Fig. 10*: The effect of *in utero* mercury exposure on the production of IL-10. Mice were exposed *in utero* to mercury and then injected as adults with DNP-KLH. Six weeks later, the splenic lymphocytes were harvested and the production of IL-10 in media alone or in response to treatment with ConA or DNP-KLH was measured. The data are shown as the mean optical density at 450nm +/- S.D. Significant differences of $p<0.05$ using Student's t-test are indicated by an asterisk.**

We did find gender-specific effects in the production of IL-4 and IL-10. Across culture conditions, cells from unexposed control male mice produced greater levels of IL-4 compared to unexposed control female mice (2.03±0.36 for male vs. 1.44±0.63 for female, p=0.03); however, there was no significant difference in the production of IL-4 by cells from mercury-exposed male mice compared to mercury-exposed female mice (1.94±0.51 for male vs. 1.61±0.63 for female, ANOVA, p=0.06) (Fig. 9, Appendix 4C). Cells from mercury-exposed and unexposed control male mice produced greater levels of IL-10 across culture conditions compared to exposure-matched female mice (control, 2.37±0.42 for male vs. 1.77±0.43 for female, $p<0.01$, and mercury, 2.31±0.37 for male vs. 1.87±0.22 for female, $p<0.01$) (Fig. 10, Appendix 4D). When cells were cultured in media alone, IL-4 production was significantly greater for mercury-exposed male mice

compared to mercury-exposed female mice (1.82±0.49 for male vs. 1.28±0.24 for female, p=0.01) (Fig. 9, Appendix 4C). The production of IL-10 was greater for mercury-exposed male mice in response to ConA compared to mercury-exposed female mice (2.38±0.45 for male vs. 1.94±0.24 for female, p=0.03), and there was greater IL-10 production in response to ConA by cells from unexposed control male mice compared to unexposed control female mice (2.26±0.02 for male vs. 1.53±0.11 for female, p=0.051) (Fig. 10, Appendix 4D). In response to DNP-KLH, there were greater levels of both IL-4 and IL-10 produced by cells from mercury-exposed male mice compared to mercury-exposed female mice (IL-4, 1.74±0.43 for male vs. 1.22±0.33 for female, p=0.02, IL-10, 2.29±0.30 for male vs. 1.79±0.12 for female, p<0.01), as well as a trend for greater IL-4 production in response to DNP-KLH in unexposed control male mice compared to unexposed control female mice (1.93±0.39 for male vs. 1.14±0.06 for female, p=0.07) (Fig. 9,10, Appendix 4C, 4D). In conclusion, our data show that as a result of gestational exposure to mercury, the production of the T_H2 cytokines may be modulated. We showed that there were gender-specific effects in the production of both IL-4 and IL-10.

Effect of in utero mercury exposure on in vitro DNP-KLH-specific immunoglobulin production

The effects of exposure to mercury during gestation on immune responses to antigen were analyzed by measuring the production of DNP-KLH-specific IgM, IgG (IgG1, IgG2a, and IgG2b), and IgE after immunization with the antigen DNP-KLH.

No differences were observed across culture conditions in the production of DNP-KLH-specific IgM by cells from mercury-exposed mice compared to unexposed control mice (0.55±0.37 for mercury vs. 0.56±0.18 for control, ANOVA, p=0.91) (Fig. 11, Appendix 5A). There was no difference in DNP-KLH-specific IgM production by cells from unexposed control male mice compared to mercury-exposed male mice when cultured in media alone (0.54±0.04 for control vs. 0.44±0.12 for mercury, ANOVA, p=0.22); similarly, no differences were found in the production of DNP-KLH-specific IgM after culture in media alone by cells from mercury-exposed female mice compared to unexposed control female mice (0.39±0.08 for mercury vs. 0.50±0.14 for control, ANOVA, p=0.17) (Fig. 11, Appendix 5A). No significant differences were observed in the production of DNP-KLH-specific IgM in response to LPS or DNP-KLH by cells from mercury-exposed mice compared to unexposed control mice (LPS, 0.84±0.52 for mercury vs. 0.68±0.24 for control, ANOVA, p=0.20, and DNP-KLH, 0.39±0.12 for mercury vs. 0.47±0.14 for control, ANOVA, p=0.51) (Fig. 11, Appendix 5A).

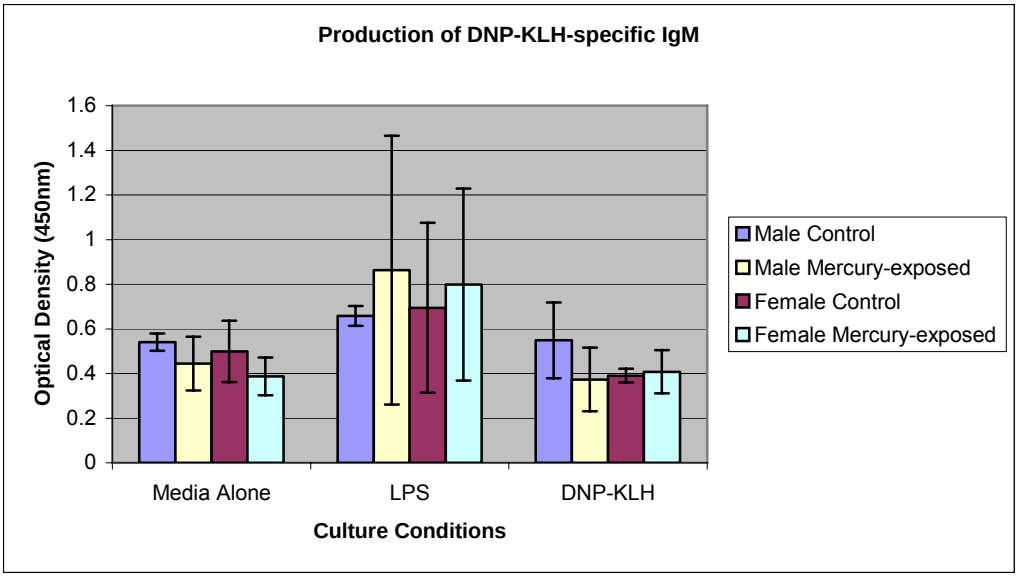

***Fig. 11*: The effect of *in utero* mercury exposure on the production of the immunoglobulin IgM to specific antigen, DNP-KLH. Mice were exposed *in utero* to mercury and then injected with DNP-KLH as adults. *In vitro* production of DNP-KLH-specific IgM in response to media alone or treatment with LPS or DNP-KLH was measured using ELISA. Data are shown as the mean optical density at 450nm +/- S.D.**

The production of DNP-KLH-specific IgG was measured. Across culture conditions, there was no difference in the production of DNP-KLH-specific IgG by cells from mercury-exposed mice compared to unexposed control mice (1.37±0.19 for mercury vs. 1.35±0.22 for control, ANOVA, p=0.94) (Fig. 12, Appendix 5C). When cells were cultured in media alone, there was a greater production of DNP-KLH-specific IgG in mercury-exposed male mice compared to unexposed control male mice (1.53±0.18 for mercury vs. 1.27±0.06 for control, $p<0.01$); however, there were no differences in the production of DNP-KLH-specific IgG in mercury-exposed female mice compared to unexposed control female mice (1.29±0.19 for mercury vs. 1.23±0.09 for control, ANOVA, p=0.62) (Fig. 12, Appendix 5C). We found that there was no difference in the production of DNP-KLH-specific IgG in response to LPS in unexposed control mice

compared to mercury-exposed mice (1.45±0.35 for control vs. 1.42±0.19 for mercury, ANOVA, p=0.55) (Fig. 12, Appendix 5C). When cells were cultured in the presence of DNP-KLH, there was no difference in the production of DNP-KLH-specific IgG in unexposed control mice compared to mercury-exposed mice (1.36±0.13 for control vs. 1.27±0.14 for mercury, ANOVA, p=0.16) (Fig. 12, Appendix 5C).

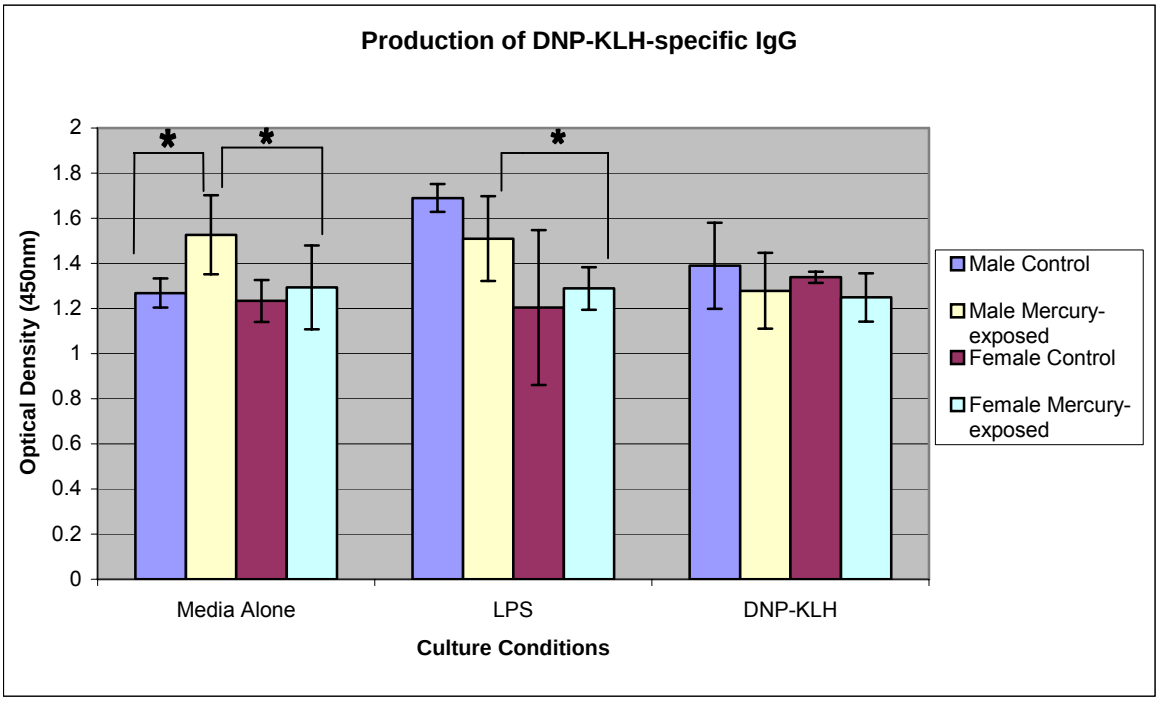

Fig. 12: The effect of in utero mercury exposure on the production of the immunoglobulin IgG to specific antigen, DNP-KLH. Mice were exposed in utero to mercury and then injected with DNP-KLH as adults. In vitro production of DNP-KLH-specific IgG in response to media alone or to treatment with LPS or DNP-KLH was measured. Data are shown as the mean optical density at 450nm +/- S.D. Significant differences of p<0.05 using Student's t-test are indicated by an asterisk.

Significant gender differences in the production of DNP-KLH-specific IgG were also found. Across culture conditions, cells from mercury-exposed male mice produced greater levels of DNP-KLH-specific IgG compared to mercury-exposed female mice

(1.44±0.21 for male vs. 1.28±0.13 for female, $p<0.01$); however, there was no difference observed in the production of DNP-KLH-specific IgG by cells from unexposed control male mice compared to unexposed control female mice (1.45±0.22 for male vs. 1.26±0.19 for female, ANOVA, p=0.06) (Fig. 12, Appendix 5C). After cells were cultured in media alone, the production of DNP-KLH-specific IgG was greater in mercury-exposed male mice compared to mercury-exposed female mice (1.53±0.18 for male vs. 1.29±0.19 for female, p=0.03) (Fig. 12, Appendix 5C). In response to LPS, mercury-exposed male mice produced greater levels of DNP-KLH-specific IgG compared to mercury-exposed female mice (1.51±0.19 for male vs. 1.29±0.09 for female, p=0.01) (Fig. 12, Appendix 5C). However, in response to DNP-KLH, no differences were found in the production of DNP-KLH-specific IgG by cells from mercury-exposed male mice compared to mercury-exposed female mice (1.28±0.17 for male vs. 1.25±0.11 for female, p=0.69) (Fig. 12, Appendix 5C).

Since some studies have found increased production of IgE after mercury exposure, the production of DNP-KLH-specific IgE was measured. There was no significant difference across culture conditions between mercury-exposed mice and unexposed control mice (0.32±0.11 for mercury vs. 0.39±0.21 for control, ANOVA, p=0.14) (Fig. 13, Appendix 5B). There were no significant differences observed in the production of DNP-KLH-specific IgE by cells from mercury-exposed mice compared to unexposed control mice when cells were cultured in media alone (0.32±0.08 for mercury vs. 0.33±0.04 for control, ANOVA, p=0.81) (Fig. 13, Appendix 5B). Similarly, there were no differences observed in the production of DNP-KLH-specific IgE in response to

LPS in mercury-exposed mice compared to unexposed control mice (0.34±0.16 for mercury vs. 0.50±0.34 for control, ANOVA, p=0.18) (Fig. 13, Appendix 5B). In response to DNP-KLH, there was no significant difference in the production of DNP-KLH-specific IgE by cells from mercury-exposed male mice compared to unexposed control male mice (0.25±0.03 for mercury vs. 0.33±0.09 for control, p=0.25); similarly, there was no difference in the production of DNP-KLH-specific IgE in response to DNP-KLH by cells from mercury-exposed female mice compared to unexposed control female mice (0.37±0.06 for mercury vs. 0.33±0.10 for control, ANOVA, p=0.45) (Fig. 13, Appendix 5B).

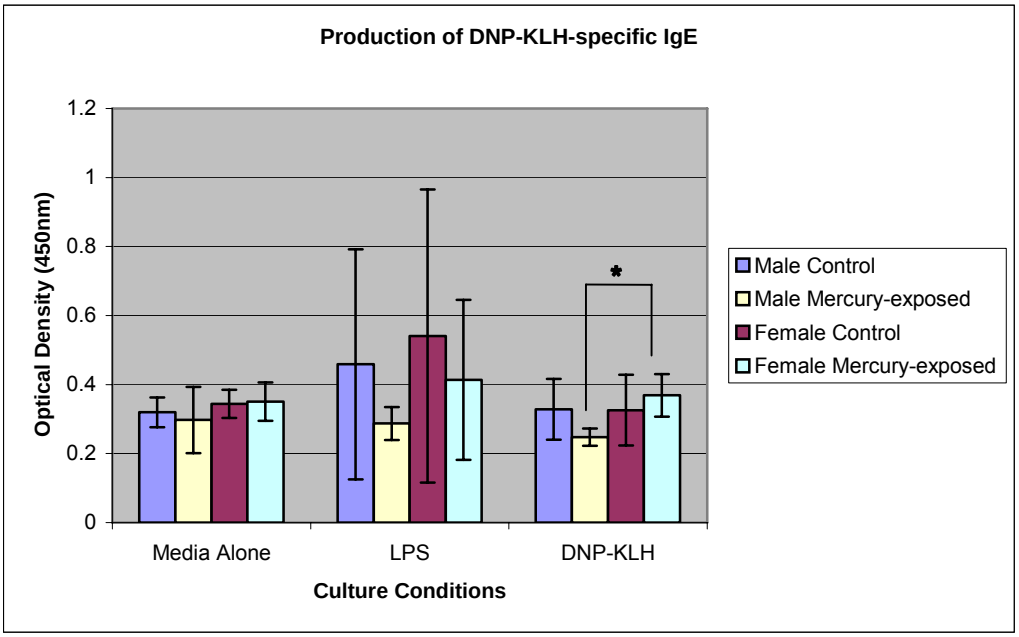

Fig. 13: The effect of in utero mercury exposure on the production of the immunoglobulin IgE to specific antigen, DNP-KLH. Mice were exposed in utero to mercury and then injected with DNP-KLH as adults. Production of DNP-KLH-specific IgE in vitro in response to media alone or treatment with LPS or DNP-KLH was measured using ELISA. Data are shown as mean optical density at 450nm +/- S.D. Significant differences of p<0.05 using Student's t-test are indicated by an asterisk.

However, we did observe gender-specific differences in the production of IgE in this study. When cultured in the presence of DNP-KLH, we found higher levels of DNP-KLH-specific IgE was produced by cells from mercury-exposed female mice compared to mercury-exposed male mice (0.37 ± 0.06 for female vs. 0.25 ± 0.03 for male, $p<0.01$) (Fig. 13, Appendix 5B). In contrast, no differences were found in the production of DNP-KLH-specific IgE in mercury-exposed female mice compared to mercury-exposed male mice for cells cultured in media alone or in response to LPS (media alone, 0.35 ± 0.06 for female vs. 0.30 ± 0.10 for male, ANOVA, $p=0.19$, and LPS, 0.41 ± 0.23 for female vs. 0.29 ± 0.05 for male ANOVA, $p=0.26$) (Fig. 13, 5B).

DISCUSSION

Similar to previous research by *Pilones et al*. and *Silva et al*., this study found persistent effects into adulthood of gestational exposure to mercury on the immune system in mice (1, 11). Similar to these studies, which found no differences in splenic cellularity as a result of gestational mercury exposure, we also did not find significant differences in splenic cellularity as a result of *in utero* exposure to mercury (1, 11).

The phenotypes of the splenocytes in response to DNP-KLH were determined using flow cytometry. This study found a reduced percentage and total number of CD4+CD25+ cells in mercury-exposed female mice compared to unexposed control female mice, but there was also a slightly greater percentage and a greater number of total CD4+CD25+ cells in mercury-exposed male mice compared to unexposed control male mice. Similarly, *Pilones et al*. previously found reduced numbers of CD4+ cells from the spleen co-expressing CD25+ in 10-week-old female mice exposed to mercury *in utero*, and there was also a trend for increased numbers of CD4+ cells from the spleen co-expressing CD25+ in mercury-exposed male mice (1). One reason that the reduction in the numbers of CD4+CD25+ cells, which are activated regulatory T cells, poses a health risk of autoimmunity is because reduced numbers of CD4+CD25+ cells are associated with increased T helper cell activity and the development of autoimmunity (30,31). In the case of response to a foreign antigen, fewer T regulatory cells could lead to enhanced immune responses. There was also a reduced percentage and reduced total cell number of B220+ cells, which are immunoglobulin-producing B lymphocytes, in mercury-exposed female mice compared to unexposed control female mice, suggesting that

mercury exposure could lead to decreased immunoglobulin production. The percentage and total cell numbers of NK1.1+ cells, natural killer cells, and GITTR+ cells, the expression of which indicated cells that can abrogate the inhibitory activity of regulatory T cells, were both reduced in mercury-exposed female mice compared to unexposed control female mice. These data suggest that intrauterine mercury exposure may have negative long-term effects on the immune response to an antigen, in this case DNP-KLH, and possibly on the immune response to infectious agents because natural killer cells are involved in innate immunity to intracellular pathogens. Differences in GITTR expression could affect T cell regulation. There were gender differences in the effect of intrauterine mercury exposure as there was a slightly greater percentage and total cell number of NK1.1+ cells in mercury-exposed male mice compared to unexposed control male mice, and there was a greater percentage and total cell number of GITTR+ cells in mercury-exposed male mice compared to unexposed control male mice. As a result of gestational mercury exposure, the percentages and total numbers of cells were generally reduced in female mice, but were slightly greater or greater in male mice.

This study found increased proliferative response of splenocytes overall across culture conditions, from female mice across culture conditions, from male mice across culture conditions, to ConA, to LPS, and to LPS specifically in females after gestational exposure to mercury. These data fit in with the reduced numbers of T regulatory CD4+CD25+ cells and decreased GITTR because both cell types could lead to enhanced B cell activation in female mice. Thus, although there are decreased numbers of B220+ cells in mercury-exposed female mice, these B cells show enhanced proliferative

responses. Increased CD4+CD25+ activated effector cells without increased GITTR+ cells in male mice could contribute to increased T cell activation. These results contrasted greatly with the results of *Silva et al*., which found no effect of gestational mercury exposure on the proliferative response of splenocytes in response to ConA (11). Differences in the results of this study and those of *Silva et al.* may be the result of differences in the routes of mercury exposure, or in the ages of the mice. Mice in our study were 44-weeks-old, and the mice in the study by *Silva et al.* were no older than 60 days of age (11). In the study by *Silva et al.*, a different protocol for mercury exposure was used, sub-cutaneous injection of pregnant dams with 200μg/kg body weight of mercuric chloride every other day for 11 days starting at g.d. 5. Although the amount of mercury in the exposure used in the study by *Silva et al.* (11) was comparable to the concentration of mercury used for exposure in the present study, calculated as approximately 208μg/kg/day (1), differences in the route and method of exposure from the present study would likely result in different absorption rates and target organs (4, 23). *Silva et al.* did note also that the effects of intrauterine mercury exposure on the immune system varied with the age of the mice (11). Similar to the present study, *Pilones et al.* found increased proliferative response of splenocytes in response to LPS in female mice as a result of *in utero* exposure to mercury; however, they also found an increased proliferative response of splenocytes in response to ConA in specifically female mice due to gestational mercury exposure, which differed from the results of our study (1). They also found increased proliferative responses of splenocytes in response to ConA or LPS specifically in male mice after gestational exposure to mercury; however, we only found overall, not within sex for male mice, increased proliferative responses in

response to ConA or LPS as a result of *in utero* exposure to mercury. Differences in the ages of the mice, in the study by *Pilones et al.* compared to our study, may explain these results (1). Mice in our study were 44-weeks-old, and mice in the study by *Pilones et al.* were 10-weeks-old (1).

To determine the effects on the T_H1 and T_H2 immune responses, the levels of the T_H1 cytokines IL-2 and IFN-γ and the T_H2 cytokines IL-4 and IL-10 were measured (1,6,11). These cytokines have both effector and inhibitory functions within the immune system (2, 3) (Fig. 14). Based on previous studies, we expected that as a result of mercury exposure, we would find modulations in the production of both T_H1 and T_H2 cytokines with possible gender-specific effects.

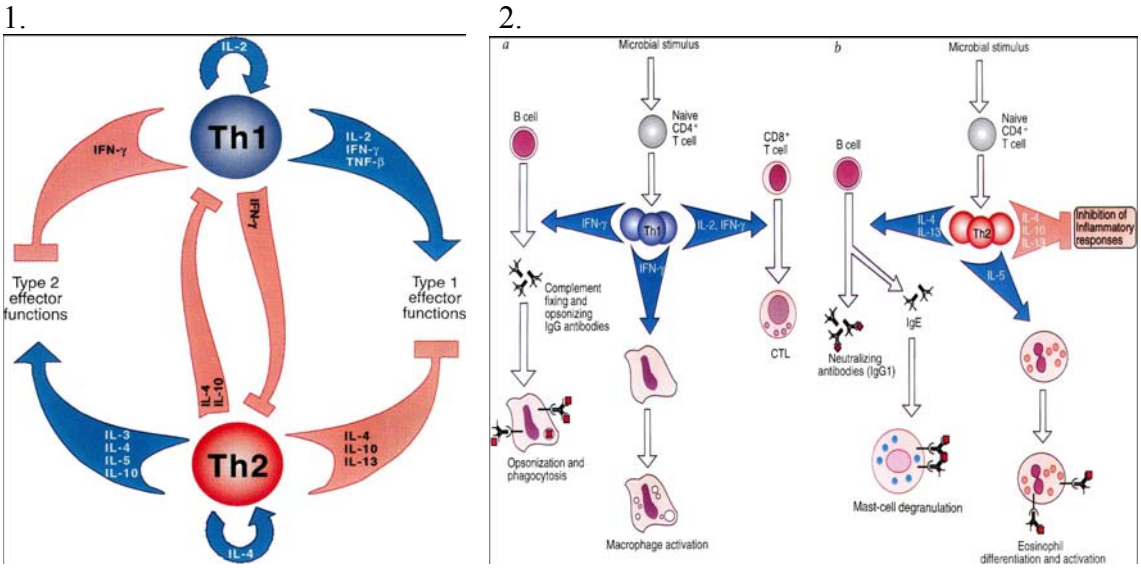

***Fig. 14*: The generalized functions of T_H1 and T_H2 cytokines. 1: The effector functions of the cytokines are shown in blue, and the inhibitory functions of the cytokines are shown in red. 2a: The function of T_H1 cytokines produced in response to a microbial antigen is shown. 2b: The effects of T_H2 cytokines produced as a result of the presence of an antigen (2).**

No differences were observed in the production of the T_H1 cytokines IL-2 or IFN-γ. Although *Pilones et al*. also found no changes in IL-2 production due to mercury exposure, they also found greater production of IFN- γ by splenic lymphocytes in response to ConA in both male and female mice as a result of intrauterine mercury exposure, which differed from the results of our study (1). These results also contrast results from a study by *Silva et al*., in which there was an increase in the production of IFN-γ in response to ConA at day 14 after parturition in mercury-exposed mice and a decrease in IL-2 production in response to ConA at day 21 after parturition in mercury-exposed mice (11).

In this study, there were no significant differences found in the production of the T_H2 cytokines IL-4 or IL-10 resulting from intrauterine exposure to mercury. In contrast, *Pilones et al.* found a trend for the increased production of IL-10 in response to ConA due to gestational mercury exposure as well as increased production of IL-4 in response to ConA in male and female mice resulting from *in utero* exposure to mercury (1). The results from *Silva et al*. also differed from this study because they found an increased production of IL-10 in response to ConA in mice at day 21 after parturition as a result of mercury exposure, and then found decreased production of IL-10 in response to ConA in female mice at day 60 after parturition due to mercury exposure during gestation (11).

The present study also examined the effects of intrauterine mercury exposure on the immune responses to an antigen. There were no differences in the responses of DNP-KLH-immunized mice to a second exposure to the antigen resulting from gestational

exposure to mercury. There was, however, a gender-specific difference in that there was increased proliferative response of splenocytes in mercury-exposed male mice in response to DNP-KLH compared to mercury-exposed female mice. However, while there were no differences observed in the production of IL-2, IFN-γ, IL-4, or IL-10 in response to DNP-KLH as a result of gestational mercury exposure, we did find that there were gender-specific effects. The production of IFN-γ, IL-4, and IL-10 was greater in mercury-exposed male mice compared to mercury-exposed female mice in response to DNP-KLH, but we also noted that there was also a tendency for the increased production of IL-4 in response to DNP-KLH in unexposed control male mice compared to unexposed control female mice.

To analyze whether modulations in antigen-specific immunoglobulins resulted from gestational mercury exposure, ELISA was used to determine levels of DNP-KLH-specific IgM, IgG (IgG1, IgG2a, and IgG2b), and IgE in mercury-exposed and unexposed control DNP-KLH-immunized mice. Interestingly, there were also no differences found in the production of DNP-KLH-specific IgM in response to LPS in mice as a result of gestational exposure to mercury even though there were increased proliferative responses of splenocytes in response to LPS overall and by cells from female mice as a result of exposure to mercury *in utero*. This suggests that while mercury exposure will lead to enhanced polyclonal B cell activation, it will not necessarily lead to enhanced numbers of antigen-specific B cell or immunoglobulin production. In contrast to the results of DNP-KLH-specific IgM production, the production of DNP-KLH-specific IgG by cells cultured in media alone was increased in male mice as a result of mercury exposure

during gestation. No differences were observed in the production of DNP-KLH-specific IgE as a result of gestational exposure to mercury, but there was a gender-specific difference in response to DNP-KLH, in which there was greater production of DNP-KLH-specific IgE in mercury-exposed female mice compared to mercury-exposed male mice, even though there was a greater proliferative response of splenocytes in response to DNP-KLH in mercury-exposed male mice compared to mercury-exposed female mice.

IL-4 is known to induce the switching of B cells to the IgE isotype (3). In this study, although there was increased production of IL-4 in cells cultured in media alone and in response to DNP-KLH in mercury-exposed male mice compared to mercury-exposed female mice, and there was a trend for increased production of IL-4 in cells in response to DNP-KLH in unexposed control male mice compared to unexposed control female mice, we did not find an effect on the production of DNP-KLH-specific IgE. In fact, the production of DNP-KLH-specific IgE in response to DNP-KLH was greater in mercury-exposed female mice compared to mercury-exposed male mice.

Gender-specific differences were observed in the proliferative response of splenocytes, the production of cytokines, and in the production of DNP-KLH-specific immunoglobulins. The splenocytes of male mice had greater proliferative responses than the splenocytes of female mice after cells were cultured in media alone in unexposed control mice and in mercury-exposed mice and in response to DNP-KLH in mercury-exposed mice. Male mice also had increased IFN-γ and IL-4 production compared to female mice in mercury-exposed mice by cells cultured in media alone and in response to

DNP-KLH; however, there was a trend for greater production of IL-4 in cells in response to DNP-KLH in unexposed control male mice compared to unexposed control female mice. In contrast, the production of IL-10 was greater in response to ConA in mercury-exposed male mice compared to mercury-exposed female mice, although there was also increased IL-10 production in response to ConA in unexposed control male mice compared to unexposed control female mice. Splenocytes of male mice also showed increased IL-10 production in response to DNP-KLH compared to female mice after gestational mercury exposure. The production of IL-10 was also greater in cells cultured in media alone in mercury-exposed male mice compared to mercury-exposed female mice, although there was also increased production of IL-10 by cells from unexposed control male mice compared to unexposed control female mice. These results show greater effects in male mice compared to female mice, especially after gestational exposure to mercury, which contrasts with the results of *Pilones et al*., which found greater effects in female mice in proliferative response and no gender differences in the production of IL-2, IFN-γ, IL-4, or IL-10 (1). Similar to this study, *Silva et al*. found gender effects in 60-day-old mice (11). That study found an inhibitory effect on the production of IL-10 in mercury-exposed female mice, and in male mice, *Silva et al*. found a stimulatory effect of gestational mercury exposure on the production of IFN-γ, IL-10, and IL-4, and this study also found greater production of IFN-γ, IL-10, and IL-4 by splenocytes of mercury-exposed male mice compared to mercury-exposed female mice (11).

Gender differences were also found in the production of DNP-KLH-specific IgG and IgE, but not IgM. Again, mercury-exposed male mice produced greater amounts of DNP-KLH-specific IgG by cells across culture conditions, by cells cultured in media alone, and by cells in response to LPS compared to mercury-exposed female mice; however, the production of DNP-KLH-specific IgE was greater in response to DNP-KLH in mercury-exposed female mice compared to mercury-exposed male mice. It is interesting to speculate that the gender differences observed in this study and others might be the result of mercury's potential role as an endocrine disruptor. Mercury has been shown to interact with ER_α (20), and ER_α and estrogen have roles in the normal development of the thymus (27, 28). ER_α is expressed on immune cells and can interact with estrogen and lead to the activation of the cells (32). More research is needed to better understand when in development mercury possibly causes the greatest alterations as a result of interaction with ER_α, and which alterations specifically occur as a result of the interaction between mercury and ER_α.

In summary, we have found that exposure to mercuric chloride *in utero* resulted in altered immune cell phenotypes and immune function that lasted into adulthood. Alterations were also observed in the response to the antigen, DNP-KLH, as a result of gestational exposure to mercury. The alterations were observed in splenocyte phenotype and in gender-specific effects in the production of DNP-KLH-specific immunoglobulins, the proliferative response of splenocytes, and in the production of cytokines. Gender-specific effects were observed as a result of intrauterine exposure to mercury, with a general increased effect in male mice compared to female mice. The results present the

potential long-term health risks of altered immune response to infectious agents or of the induction of autoimmune responses, and the results emphasize the importance of the risk of gestational exposure to xenobiotics. The experiment should be repeated in order to verify the data, and further research is needed to better understand the mechanisms of immunotoxity resulting from gestational exposure to mercury.

LITERATURE CITED

1. Pilones, K., Tatum, A., Gavalchin, J. (In print). Gestational exposure to mercury leads to persistent changes in T-cell phenotype and function in adult DBF_1 mice.
2. Abbas, A.K., Murphy, M.K., and Sher, A. (1996). Functional diversity of helper T lymphocytes. *Nature*. 383, 787-793.
3. Parham, P. (2000). *The Immune System*. New York: Garland Publishing/Elsevier Science Ltd.
4. Agency for Toxic Substances and Disease Registry (ATSDR). (1999). Toxicological profile for mercury. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.
5. Ask, K., Akesson, A., Berglund, M., and Vahter, M. (2002). Inorganic mercury and methylmercury in placentas in of Swedish women. *Environmental Health Perspectives*. 110, 523-526.
6. Pilones, K., Lai, Z., Gavalchin, J. (2007). Prenatal $HgCl_2$ exposure alters fetal cell phenotypes. *Journal of Immunotoxicology*. 4, 295-301.
7. Steuerwald, U., Weihe, P., Jorgensen, P.J., Bjerve, K., Brock, J., Heinzow, B., Budtz-Jorgensen, E., and Grandjean, P. (2000). Maternal seafood diet, methylmercury exposure, and neonatal neurologic function. *Journal of Pediatrics*. 136, 599-605.

8. Hodgson, S., Nieuwenhuijsen, M.J., Elliott, P., and Jarup, L. (2007). Kidney Disease Mortality and Environmental Exposure to Mercury. *American Journal of Epidemiology*. 165, 72-77.
9. Oskarsson, A., Schültz, A., Skerfving, S., Hallén, I.P., Ohlin, B., and Lagerkvist, B.J. (1996). Total and inorganic mercury in breast milk in relation to fish consumption and amalgam in lactating women. *Archives of Environmental Health*. 51, 234-241.
10. Debes, F., Budtz-Jorgensen, E., Weihe, P., White, R.F., and Grandjean, P. (2006). Impact of prenatal methylmercury exposure on neurobehavioral function at age 14 years. *Neurotoxicology and Teratology*. 28, 536-547.
11. Silva, I.A., El Nabawi, M., Hoover, D., and Silbergeld, E.K. (2005). Prenatal $HgCl_2$ exposure in BALB/c mice: gender-specific effects on the ontogeny of the immune system. *Developmental and Comparative Immunology*. 29, 171-183.
12. DNP-KLH (Keyhole Limpet Hemocyanin). (2008) Product Detail. Biosearch Technologies. <http://www.biosearchtechnologies.com/products/details.asp?id=288&catID=>.
13. Yan, F., Yan, J., Wang, Y., and Chen, D. (2008). Site environmental assessment to an old paint factory in China. *Environmental Monitoring and Assessment*. 139, 93-106.
14. Costa, C., and Jesus-Rydin, C. (2001). Site investigation on heavy metals contaminated ground in Esatrreja-Portugal. *Engineering Geology*. 60, 39-47.
15. Drinking water contaminants. (2008). U.S. Environmental Protection Agency. <http://www.epa.gov/safewater/contaminants/index.html#listmcl>.

16. Risher, J.F., Murray, H.E., and Prince, G.R. (2002). Organic mercury compounds: human exposure and its relevance to public health. *Toxicology and Industrial Health*. 18, 109-160.

17. Stauding, K.C., and Roth, V.S. (1998). Occupational lead poisoning; includes patients handouts on lead and health. *American Family Physician*. 57, 719.

18. Guallar, E., Sanz-Gallardo, M.I., van't Veer, P., Bode, P., *et al*. (2002). Mercury, fish oils, and the risk of myocardial infarction. *The New England Journal of Medicine.* 347, 1747-1754.

19. Oliveira, E.M., Vassallo, D.V., Sarkis, J.J.F., and Mill, J.G. (1994). Mercury effects on the contractile activity of isolated heart muscle. *Toxicology and Applied Pharmacology*. 128, 86-91.

20. Martin, M.B., Reiter, R., Pham, T., Avellanet, Y.R., Camara, J., Lahm, M., Pentecost, E., Pratap, K., Gilmore, B.A., Divekar, S., Dagata, R.S., Bull, J.L., and Stoica, A. (2003). Estrogen-like activity of metals in Mcf-7 breast cancer cells. *Endocrinology*. 144, 2425-2436.

21. Darbre, P.D. (2006). Metalloestrogens: an emerging class of inorganic xenoestrogens with potential to add to the oestrogenic burden of the human breast. *Journal of Applied Toxicology*. 26, 191-197.

22. Brzozowski, A.M., Pike, A.C.W., Dauter, Z., Hubbard, R.E., Bonn, T, Engström, O., Öhman, L., Greene, G.L., Gustafsson, J., and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*. 389, 753-758.

23. Tchounwou, P.B., Ayensu, W.K., Ninashvili, N., and Sutton, D. (2003). Environmental exposure to mercury and its toxicopathologic implications for public health. *Environmental Toxicology*. 18, 149-175.

24. Christensen, M.M. (1996). Histochemical localization of autometallographically detectable mercury in tissues of the immune system from mice exposed to mercuric chloride. *Histochemical Journal*. 28, 217-225.

25. Planas-Bohne, F., Taylor, D.M., and Walser, R. (1985) The influence of administered mass on the subcellular distribution and binding of mercury in rat liver and kidney. *Archives of Toxicology*. 56, 242-246.

26. Yoshida, M. (2002). Placental to fetal transfer of mercury and fetotoxicity. *Tohoku Journal of Experimental Medicine*. 196, 79-88.

27. Staples, J.E., Gasiewicz, T.A., Fiore, N.C., Lubahn, D.B., Korach, K.S., and Silverstone, A.E. (1999). Estrogen receptor-α is necessary in thymic development and estradiol-induced thymic alterations. *The Journal of Immunology*. 163, 4168-4174.

28. Erlandsson, M.C., Ohlsson, C., Gustafsson, J., and Carlsten, H. (2001). Role of oestrogen receptors α and β in immune organ development and in oestrogen-mediated effects on thymus. *Immunology*. 103, 17-25.

29. Guo, T.L., Miller, M.A., Shapiro, I.M., Shenker, B.J. (1998). Mercuric chloride induces apoptosis in human T lymphocytes: evidence of mitochondrial dysfunction. *Toxicology and Applied Pharmacology*. 153, 250-257.

30. Layland, L.E., Wulferink, M., Dierkes, S., and Gleichmann, E. (2004). Drug-induced autoantibody formation in mice: triggering by primed CD4+CD25- T

cells, prevention by primed CD4+CD25+ T cells. *European Journal of Immunology*. 34, 36-46.

31. Sakaguchi, S., Sakaguchi, N., Shimizu, J., Yamazaki, S., Sakihama, T., Itoh, M., Kuniyasu, Y., Nomura, T., Toda, M., and Takahashi, T. (2001). Immunologic tolerance maintained by CD25+CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunological Reviews*. 182, 18-32.
32. Maret, A., Coudert, J.D., Garidou, L., Foucras, G., Gourdy, P., Krust, A., Dupont, S., Chambon, P., Druet, P., Bayard, F., and Guéry, J. (2003). Estradiol enhances primary antigen-specific CD4 T cell responses and Th1 development *in vivo*. Essential role of estrogen receptor α expression in hematopoietic cells. *European Journal of Immunology*. 33, 512-521.
33. (2008). Dental filling facts. American Dental Association. 3,6.
34. (1997). Mercury Study Report to Congress. From, Environmental Protection Agency. Volume I: Executive Summary.
35. Schober, S.E., Sinks, T.H., Jones, R.L., et al. (2003). Blood mercury levels in US children and women of childbearing age, 1999-2000. *Journal of the American Medical Association*. 289, 1667- 1674.
36. Bilrha, H., Roy, R., Moreau, B., Belles-Isles, M., Dewailly, E., and Ayotte, P. (2003). *In vitro* activation of cord blood mononuclear cells and cytokine production in a remote coastal population exposed to organochlorines and methyl mercury. *Environmental Health Perspectives*. 111, 1952-1957.

37. Belles-Isles, M., Ayotte, P., Dewailly, E., Weber, J., and Roy, R. (2002). Cord blood lymphocyte functions in newborns from a remote maritime population exposed to organochlorines and methylmercury. *Journal of Toxicology and Environmental Health, Part A.* 65, 165-182.

APPENDICES

Appendix 1: Abbreviations

MC1	Male Control 1
MC2	Male Control 2
MC3	Male Control 3
MT1	Male Treatment 1
MT2	Male Treatment 2
MT3	Male Treatment 3
MT4	Male Treatment 4
MT5	Male Treatment 5
MT6	Male Treatment 6
MT7	Male Treatment 7
MT8	Male Treatment 8
MT9	Male Treatment 9

FC1	Female Control 1
FC2	Female Control 2
FC3	Female Control 3
FT1	Female Treatment 1
FT2	Female Treatment 2
FT3	Female Treatment 3
FT4	Female Treatment 4
FT5	Female Treatment 5
FT6	Female Treatment 6

Media Alone (Blank)	B
Concanavalin-A	ConA
Lipopolysaccharide	LPS
2,4-dinitrophenyl-keyhole limpet hemocyanin	DNP-KLH
Exposure group (Mercury-exposed or control)	TMT

Appendix 2: Splenocyte Numbers

Male:

Sample	Number of Cells
MC1	10,400,000
MC2	44,000,000
MC3	12,000,000
Mean	**22,133,333**
Std. Dev.	**18953979.35**

Female:

Sample	Number of Cells
FC1	54,400,000
FC2	110,400,000
FC3	21,600,000
Mean	**62,133,333**
Std. Dev.	**44902264.23**

Sample	Number of Cells
MT1	15,500,000
MT2	80,000,000
MT3	90,000,000
MT4	44,000,000
MT5	44,000,000
MT6	93,000,000
MT7	85,000,000
MT8	43,000,000
MT9	58,000,000
Mean	**61,388,889**
Std. Dev.	**26883751.8**

Sample	Number of Cells
FT1	86,400,000
FT2	46,400,000
FT3	64,000,000
FT4	51,200,000
FT5	28,800,000
FT6	73,600,000
Mean	**58,400,000**
Std. Dev.	**20583488.53**

ANOVA

Effect	Num DF	Den DF	F Value	Pr>F
Sex	1	17	1.95	0.1807
TMT	1	17	1.8	0.1979
Sex*TMT	1	17	2.63	0.1233

***t*-test, Unequal Variances**

	P-value
Male Control vs. Male Treatment	**0.0389647**
Female Control vs. Female Treatment	0.90156438
Male Control vs. Female Control	0.2600584
Male Treatment vs. Female Treatment	0.81168071

Appendix 3: Cell Proliferation Assay Data

O.D. Values:

Data for Samples in Media Alone

Sample	Blank	Blank	Blank	**Blank Mean**	**Blank Std. Dev.**
MC1	0.352	0.33	0.204	**0.295333333**	
MC2	0.327	0.314	0.33	**0.323666667**	
MC3	0.314	0.266	/	**0.29**	
Mean MC				**0.303**	**0.018095426**
MT1	0.392	0.3	0.291	**0.327666667**	
MT2	0.627	0.607	0.485	**0.573**	
MT3	0.349	0.337	0.408	**0.364666667**	
MT4	0.802	0.902	0.88	**0.861333333**	
MT5	1.003	1.209	1.084	**1.098666667**	
MT6	0.328	0.399	0.34	**0.355666667**	
MT7	0.3	0.291	0.242	**0.277666667**	
MT8	0.94	0.833	0.781	**0.851333333**	
MT9	0.332	0.334	0.339	**0.335**	
Mean MT				**0.560555556**	**0.302123319**
FC1	0.281	0.259	0.259	**0.266333333**	
FC2	0.249	0.235	0.24	**0.241333333**	
FC3	0.245	0.235	0.249	**0.243**	
Mean FC				**0.250222222**	**0.013977495**
FT1	0.251	0.236	0.25	**0.245666667**	
FT2	0.261	0.188	0.241	**0.23**	
FT3	0.253	0.271	0.279	**0.267666667**	
FT4	0.285	0.275	0.274	**0.278**	
FT5	0.263	0.258	0.277	**0.266**	
FT6	0.313	0.286	0.283	**0.294**	
Mean FT				**0.263555556**	**0.022822666**

Data for Samples with ConA

Sample	ConA	ConA	ConA	**ConA mean**	**ConA Std. Dev.**
MC1	0.351	0.35	0.053	**0.251333**	
MC2	0.347	0.357	0.362	**0.355333**	
MC3	0.252	0.241	/	**0.2465**	
Mean MC				**0.284389**	**0.061487201**
MT1	0.28	0.355	0.362	**0.332333**	
MT2	0.667	0.442	0.457	**0.522**	
MT3	0.664	0.566	0.68	**0.636667**	
MT4	0.913	0.841	0.853	**0.869**	
MT5	0.767	0.883	0.882	**0.844**	
MT6	0.462	0.505	0.496	**0.487667**	
MT7	0.297	0.318	0.216	**0.277**	
MT8	0.682	0.805	0.624	**0.703667**	
MT9	0.372	0.327	0.331	**0.343333**	
Mean MT				**0.557296**	**0.220264263**
FC1	0.339	0.335	0.316	**0.33**	
FC2	0.345	0.361	0.328	**0.344667**	
FC3	0.24	0.235	0.224	**0.233**	
Mean FC				**0.302556**	**0.060681622**
FT1	0.44	0.366	0.431	**0.412333**	
FT2	0.443	0.41	0.379	**0.410667**	
FT3	0.397	0.232	0.345	**0.324667**	
FT4	0.462	0.452	0.29	**0.401333**	
FT5	0.375	0.401	0.394	**0.39**	
FT6	0.332	0.408	0.418	**0.386**	
Mean FT				**0.3875**	**0.032561566**

Data for Samples With LPS

Sample	LPS	LPS	LPS	**LPS mean**	**LPS Std. Dev.**
MC1	0.301	0.348	0.093	**0.24733333**	
MC2	0.298	0.314	0.276	**0.296**	
MC3	0.275	0.285	/	**0.28**	
Mean MC				**0.27444444**	**0.02480442**
MT1	0.385	0.373	0.377	**0.37833333**	
MT2	0.672	0.149	0.688	**0.503**	
MT3	0.624	0.578	0.663	**0.62166667**	
MT4	1.102	0.99	1.054	**1.04866667**	
MT5	1.171	1.1	1.146	**1.139**	
MT6	0.508	0.622	0.65	**0.59333333**	
MT7	0.272	0.271	0.309	**0.284**	
MT8	0.809	0.902	0.769	**0.82666667**	
MT9	0.316	0.325	0.318	**0.31966667**	
Mean MT				**0.63492593**	**0.310186224**
FC1	0.311	0.335	0.329	**0.325**	
FC2	0.371	0.327	0.266	**0.32133333**	
FC3	0.225	0.235	0.234	**0.23133333**	
Mean FC				**0.29255556**	**0.053051687**
FT1	0.339	0.332	0.382	**0.351**	
FT2	0.483	0.459	0.39	**0.444**	
FT3	0.313	0.411	0.391	**0.37166667**	
FT4	0.614	0.395	0.406	**0.47166667**	
FT5	0.418	0.424	0.349	**0.397**	
FT6	0.469	0.193	0.433	**0.365**	
Mean FT				**0.40005556**	**0.047978892**

Data for Samples With DNP-KLH

Sample	DNP-KLH	DNP-KLH	DNP-KLH	DNP-KLH mean	DNP-KLH Std. Dev.
MC1	0.313	0.365	0.139	**0.27233333**	
MC2	0.306	0.334	0.373	**0.33766667**	
MC3	0.323	0.308	/	**0.3155**	
Mean MC				**0.3085**	**0.033224405**
MT1	0.314	0.305	0.36	**0.32633333**	
MT2	0.495	0.465	0.425	**0.46166667**	
MT3	0.365	0.347	0.326	**0.346**	
MT4	0.599	0.618	0.599	**0.60533333**	
MT5	0.687	0.777	0.915	**0.793**	
MT6	0.328	0.35	0.338	**0.33866667**	
MT7	0.25	0.274	0.26	**0.26133333**	
MT8	0.573	0.731	0.766	**0.69**	
MT9	0.3	0.307	0.276	**0.29433333**	
Mean MT				**0.45740741**	**0.192792475**
FC1	0.237	0.256	0.259	**0.25066667**	
FC2	0.214	0.244	0.252	**0.23666667**	
FC3	0.236	0.235	0.231	**0.234**	
Mean FC				**0.24044444**	**0.00895255**
FT1	0.268	0.258	0.258	**0.26133333**	
FT2	0.192	0.258	0.248	**0.23266667**	
FT3	0.248	0.249	0.257	**0.25133333**	
FT4	0.238	0.26	0.255	**0.251**	
FT5	0.227	0.254	0.249	**0.24333333**	
FT6	0.269	0.279	0.23	**0.25933333**	
Mean FT				**0.24983333**	**0.010611838**

ANOVA

Effect	Num DF	Den DF	F Value	Pr>F
Sex	1	77	8.65	**0.0043**
TMT	1	77	13.8	**0.0004**
Culture Condition	3	77	1.9	0.1367
Sex*TMT	1	77	5.96	**0.0169**

Sex=F				
Effect	Num DF	Den DF	F Value	Pr>F
TMT	1	28	19.33	**0.0001**
Culture Condition	3	28	20.11	**<.0001**
TMT* Culture Condition	3	28	4.16	**0.0148**

Sex=M				
Effect	Num DF	Den DF	F Value	Pr>F
TMT	1	43	11.63	**0.0014**
Culture Condition	3	43	0.6	0.616

Media Alone					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	5.84	**0.0265**	
TMT	1	18	1.95	0.1793	
	MA TMT=C				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	4	15.98	**0.0162**
	MA TMT=ME				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	13	5.63	**0.0337**
DNP-KLH					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	7.93	**0.0114**	
TMT	1	18	1.65	0.2149	
	D TMT=C				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	4	11.74	**0.0266**
	D TMT=ME				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	13	6.77	**0.0219**

ConA					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	2.7	0.1177	
TMT	1	18	5.84	**0.0265**	
	ConA Sex=F				
	Effect	Num DF	Den DF	F Value	Pr>F
	TMT	1	7	7.98	**0.0256**
	ConA Sex=M				
	Effect	Num DF	Den DF	F Value	Pr>F
	TMT	1	10	4.24	0.0666
LPS					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	2.76	0.1139	
TMT	1	18	5.21	**0.0348**	
	LPS Sex=F				
	Effect	Num DF	Den DF	F Value	Pr>F
	TMT	1	7	9.44	**0.018**
	LPS Sex=M				
	Effect	Num DF	Den DF	F Value	Pr>F
	TMT	1	10	3.79	0.0801

Overall culture conditions: Means and Student's *t*-test, two-sample, unequal variances

Mean Control	0.282013875
Mean mercury-exposed	0.461622228
Control vs. mercury-exposed	**2.36345E-07**
Mean Male Unexposed Control	0.292583278
Mean Male Mercury-exposed	0.552546306
Male: Control vs. mercury-exposed	**7.76713E-07**
Mean Female Unexposed Control	0.271444472
Mean Female Mercury-exposed	0.325236111
Female: Control vs. Mercury-exposed	**0.011994931**

ConA and LPS: Overall means and Student's *t*-test, two-sample, unequal variances

ConA Mean Control	0.293472167
ConA Mean Mercury-exposed	0.4893778
Control vs. mercury-exposed	**0.001790803**
LPS Mean Control	0.283499998
LPS Mean Mercury-exposed	0.540977779
Control vs. mercury-exposed	**0.002171161**

Media Alone: Overall Means

MA Mean Control	0.276611111
MA Mean Mercury-exposed	0.441755556

DNP-KLH: Overall Means

DNP-KLH Mean Control	0.274472223
DNP-KLH Mean Mercury-exposed	0.374377777

Student's *t*-test, Paired

Male Control Blank vs. Male Control ConA	0.536218769
Male Control Blank vs. Male Control LPS	0.121463783
Male Control Blank vs. Male Control DNP-KLH	0.7431187
Male Treatment Blank vs. Male Treatment ConA	0.949726932
Male Treatment Blank vs. Male Treatment LPS	0.103296233
Male Treatment Blank vs. Male Treatment DNP-KLH	**0.026597965**
Female Control Blank vs. Female Control ConA	0.255695654
Female Control Blank vs. Female Control LPS	0.265953181
Female Control Blank vs. Female Control DNP-KLH	0.092445852
Female Treatment Blank vs. Female Treatment ConA	**0.001191242**
Female Treatment Blank vs. Female Treatment LPS	**0.001871638**
Female Treatment Blank vs. Female Treatment DNP-KLH	0.139621848

Student's *t*-test, Unequal Variances, Two-sample

Male Control Blank vs. Male Treatment Blank	**0.033942412**
Male Control ConA vs. Male Treatment ConA	**0.007417493**
Male Control LPS vs. Male Treatment LPS	**0.008180484**
Male Control DNP-KLH vs. Male Treatment DNP-KLH	**0.052920006**
Female Control Blank vs. Female Treatment Blank	0.318664223
Female Control ConA vs. Female Treatment ConA	0.121944879
Female Control LPS vs. Female Treatment LPS	**0.045617575**
Female Control DNP-KLH vs. Female Treatment DNP-KLH	0.22444125

Student's *t*-test, Unequal Variances, Two-sample

Male Control Blank vs. Female Control Blank	**0.018238721**
Male Control ConA vs. Female Control ConA	0.734123556
Male Control LPS vs. Female Control LPS	0.63136486
Male Control DNP-KLH vs. Female Control DNP-KLH	0.06240128
Male Treatment Blank vs. Female Treatment Blank	**0.018464283**
Male Treatment ConA vs. Female Treatment ConA	**0.050496554**
Male Treatment LPS vs. Female Treatment LPS	**0.053986144**
Male Treatment DNP-KLH vs. Female Treatment DNP-KLH	**0.012044147**

Appendix 4A: Data from ELISA Assay for Cytokines

T_H1:

IL-2 Data

O.D. Values (Male):

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.8546	0.8284	0.8153	0.8637	0.8166	0.7161	0.8556	0.787	1.3344	0.856	Overflow	2.8762
B	0.8388	1.0863	0.8224	0.7159	0.6841	0.6613	1.5173	1.3578	0.725	0.7592	0.9217	0.8933
C	0.7973	1.0763	0.7739	0.7284	0.7152	0.7395	0.7096	0.7197	1.8266	1.9573	0.8525	0.8624
D	0.8491	0.8864	0.8407	0.7436	1.0632	1.1126	0.7586	0.7311	0.7793	0.768	2.0199	1.882
E	0.9462	1.4865	1.252	0.9615	0.8316	0.7287	Overflow	2.7211	0.8366	0.8153	0.8116	0.8817
F	1.1077	1.8988	1.6516	1.3221	0.7545	0.7045	0.7117	0.6721	0.689	0.753	0.8179	0.6721
G	2.21	1.9325	1.4697	0.8665	1.9384	1.7903	0.7087	0.7092	0.6795	0.7413	0.9517	0.8491
H	1.5518	1.3626	1.5986	1.1315	0.6829	0.7345	Overflow	2.5348	0.9096	0.8418	1.0249	0.9472

Key	1	2	3	4	5	6
A	Blank	Blank	.0015625ul/mL Std.	.0015625ul/mL Std.	MC3-ConA	MC3-ConA
B	.2ul/mL Std.	.2ul/mL Std.	MC1-B	MC1-B	MC3-DNP-KLH	MC3-DNP-KLH
C	.1ul/mL Std.	.1ul/mL Std.	MC1-ConA	MC1-ConA	MT1-B	MT1-B
D	.05ul/mL Std.	.05ul/mL Std.	MC1-DNP-KLH	MC1-DNP-KLH	MT1-ConA	MT1-ConA
E	.025ul/mL Std.	.025ul/mL Std.	MC2-B	MC2-B	MT1-DNP-KLH	MT1-DNP-KLH
F	.0125ul/mL Std.	.0125ul/mL Std.	MC2-ConA	MC2-ConA	MT2-B	MT2-B
G	.00625ul/mL Std.	.00625 ul/mL Std.	MC2-DNP-KLH	MC2-DNP-KLH	MT2-ConA	MT2-ConA
H	.003125ul/mL Std.	.003125ul/mL Std.	MC3-B	MC3-B	MT2-DNP-KLH	MT2-DNP-KLH

Key	7	8	9	10	11	12
A	MT3-B	MT3-B	MT5-DNP-KLH	MT5-DNP-KLH	MT8-ConA	MT8-ConA
B	MT3-ConA	MT3-ConA	MT6-B	MT6-B	MT8-DNP-KLH	MT8-DNP-KLH
C	MT3-DNP-KLH	MT3-DNP-KLH	MT6-ConA	MT6-ConA	MT9-B	MT9-B
D	MT4-B	MT4-B	MT6-DNP-KLH	MT6-DNP-KLH	MT9-ConA	MT9-ConA
E	MT4-ConA	MT4-ConA	MT7-B	MT7-B	MT9-DNP-KLH	MT9-DNP-KLH
F	MT4-DNP-KLH	MT4-DNP-KLH	MT7-ConA	MT7-ConA		
G	MT5-B	MT5-B	MT7-DNP-KLH	MT7-DNP-KLH		
H	MT5-ConA	MT5-ConA	MT8-B	MT8-B		

Mean and Std. Dev. (Male):

Sample	Mean	Std. Dev.
MC-B	1.175916667	0.392695341
MC-ConA	1.025783333	0.399865822
MC-DNP-KLH	0.897633333	0.246971766
MT-B	0.781461111	0.063242765
MT-ConA	1.946638889	0.79060523
MT-DNP-KLH	0.8032	0.130960767

O.D. Values (Female):

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.8306	0.4151	0.4632	0.3817	0.333	0.3815	0.4974	0.6766	0.9202	0.992	0.0315	0.0318
B	2.4844	2.6611	0.5947	0.9026	0.9716	1.4945	2.3026	2.7995	0.9712	0.9842	0.0314	0.1051
C	2.2906	2.3647	2.9125	Overflow	0.804	1.013	0.6458	0.5645	2.9335	2.982	0.0293	0.0335
D	1.5897	1.3632	0.7003	1.1174	2.9942	2.5799	0.6066	0.6608	0.8548	1.1823	0.0538	0.0289
E	1.4642	1.1305	0.5565	0.8349	0.7997	0.6334	2.7672	2.6369	0.1819	0.0634	0.0366	0.0331
F	1.0764	1.26	2.5226	2.5844	0.8095	1.1221	0.5964	0.5676	0.2709	0.0666	0.0288	0.0319
G	0.954	0.9879	0.9598	1.0433	Overflow	Overflow	0.6098	0.5847	1.9	0.0638	0.0308	0.0295
H	0.567	0.5205	0.7141	0.7912	2.3176	0.49	2.7029	2.7835	0.1827	0.6442	0.0316	0.0283

Key	1	2	3	4	5	6
A	Blank	Blank	.0015625ul/mL Std.	.0015625ul/mL Std.	FC3-ConA	FC3-ConA
B	.2ul/mL Std.	.2ul/mL Std.	FC1-B	FC1-B	FC3-DNP-KLH	FC3-DNP-KLH
C	.1ul/mL Std.	.1ul/mL Std.	FC1-ConA	FC1-ConA	FT1-B	FT1-B
D	.05ul/mL Std.	.05ul/mL Std.	FC1-DNP-KLH	FC1-DNP-KLH	FT1-ConA	FT1-ConA
E	.025ul/mL Std.	.025ul/mL Std.	FC2-B	FC2-B	FT1-DNP-KLH	FT1-DNP-KLH
F	.0125ul/mL Std.	.0125ul/mL Std.	FC2-ConA	FC2-ConA	FT2-B	FT2-B
G	.00625ul/mL Std.	.00625 ul/mL Std.	FC2-DNP-KLH	FC2-DNP-KLH	FT2-ConA	FT2-ConA
H	.003125ul/mL Std.	.003125ul/mL Std.	FC3-B	FC3-B	FT2-DNP-KLH	FT2-DNP-KLH

Key	7	8	9	10	11	12
A	FT3-B	FT3-B	FT5-DNP-KLH	FT5-DNP-KLH		
B	FT3-ConA	FT3-ConA	FT6-B	FT6-B		
C	FT3-DNP-KLH	FT3-DNP-KLH	FT6-ConA	FT6-ConA		
D	FT4-B	FT4-B	FT6-DNP-KLH	FT6-DNP-KLH		
E	FT4-ConA	FT4-ConA				
F	FT4-DNP-KLH	FT4-DNP-KLH				
G	FT5-B	FT5-B				
H	FT5-ConA	FT5-ConA				

Mean and Std. Dev. (Female):

Sample	Mean	Std. Dev.
FC-B	0.732333	0.031788
FC-ConA	1.955667	1.39884
FC-DNP-KLH	1.047817	0.166979
FT-B	0.778325	0.190868
FT-ConA	2.790183	0.166896
FT-DNP-KLH	0.880358	0.313182

ANOVA

Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	77	8.65	**0.0043**	
TMT	1	77	13.8	**0.0004**	
Culture conditions	3	77	1.9	0.1367	
Sex*TMT	1	77	5.96	**0.0169**	
Sex=Female					
Effect	Num DF	Den DF	F Value	Pr>F	
TMT	1	31	14.81	**0.0006**	
Culture conditions	3	31	22.89	<.0001	
Sex=Male					
Effect	Num DF	Den DF	F Value	Pr>F	
TMT	1	30	0.71	**0.4064**	
Culture conditions	2	30	5.16	0.0119	
TMT* Culture conditions	2	30	5.42	0.0098	
TMT=Control					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	16	0.45	0.5135	
TMT=Mercury-exposed					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	43	0.45	0.5072	
Media Alone					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	17	6.85	**0.018**	
TMT	1	17	4.17	**0.0571**	
Sex*TMT	1	17	6.66	**0.0195**	
	MA TMT=Control				
	Effect	Num DF	Den DF	F Value	Pr>F

	Sex	1	4	3.8	0.1229
	MA TMT=Mercury-exposed				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	13	0	0.9637
	MA Sex=Female				
	Effect	Num DF	Den DF	F Value	Pr>F
	TMT	1	7	0.16	0.7004
	MA Sex=Male				
	Effect	Num DF	Den DF	F Value	Pr>F
	TMT	1	10	10.28	**0.0094**
DNP-KLH					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	1.1	0.3079	
TMT	1	18	1.56	0.227	
ConA					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	2.03	0.1715	
TMT	1	18	2.4	0.1389	

Across culture conditions: Means and Student's *t*-test, two-sample, unequal variances

Unexposed control mean	1.139192
Mercury-exposed mean	1.243701
Unexposed control vs. Mercury-exposed	0.600056
Female unexposed control mean	1.245272
Female mercury-exposed mean	1.343603
Female: unexposed control vs. Mercury-exposed	0.795707
Male unexposed control mean	1.033111
Male mercury-exposed mean	1.1771
Male: unexposed control vs. Mercury-exposed	0.418374

ConA: Overall means

Unexposed control mean	1.490725
Mercury-exposed mean	2.116834

DNP-KLH: Overall means

Unexposed control mean	0.972725
Mercury-exposed mean	0.834063

Student's *t*-test:

Paired

Comparison	
MC-B vs. MC-ConA	0.415211676
MC-B vs. MC-DNP-KLH	0.272350806
MT-B vs. MT-ConA	**0.002195919**
MT-B vs. MT-DNP-KLH	0.662143486

Two-sample, unequal variances

Comparison	
MC-B vs. MT-B	0.25675966
MC-ConA vs. MT-ConA	**0.03218406**
MC-DNP-KLH vs. MT-DNP-KLH	0.58192246

Paired

Comparison	
FC-B vs. FC-ConA	0.269019798
FC-B vs. FC-DNP-KLH	0.077209109
FT-B vs. FT-ConA	**3.62E-09**
FT-B vs. FT-DNP-KLH	0.514226257

Two-sample, unequal variances

Comparison	
FC-B vs. FT-B	0.58821834
FC-ConA vs. FT-ConA	0.43000993
FC-DNP-KLH vs. FT-DNP-KLH	0.33138985

Two-sample, unequal variances

Comparison	
MC-B vs. FC-B	0.23866551
MC-ConA vs. FC-ConA	0.36957879
MC-DNP-KLH vs. FC-DNP-KLH	0.43848045
MT-B vs. FT-B	0.97032553
MT-ConA vs. FT-ConA	**0.01267557**
MT-DNP-KLH vs. FT-DNP-KLH	0.58807528

Appendix 4B: Data from ELISA Assay for Cytokines

T_H1:

IFN-γ Data:

O.D. Values (Male):

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.8622	1.2872	1.618	1.3927	1.3805	1.8009	2.3234	1.9286	2.7398	2.3604	2.6189	2.1018
B	1.0778	2.5293	2.3262	1.8484	1.6865	2.666	2.9796	Overflow	1.9057	Overflow	Overflow	Overflow
C	1.1709	2.1494	2.165	2.1306	2.4464	2.9683	Overflow	2.8361	Overflow	Overflow	Overflow	2.6983
D	0.71	2.1875	2.4569	2.751	2.3206	Overflow	Overflow	2.7971	Overflow	Overflow	Overflow	1.6854
E	0.7618	2.1962	Overflow	2.944	Overflow	Overflow	2.7209	2.6286	Overflow	Overflow	Overflow	Overflow
F	1.2469	2.6404	Overflow	2.6927	Overflow	2.5837	2.9089	Overflow	2.8248	Overflow	2.1633	2.2667
G	0.9577	2.5701	Overflow	2.6861	Overflow	2.9595	2.6137	Overflow	2.6692	Overflow	Overflow	2.9358
H	0.4781	1.3933	1.8634	1.4157	1.1041	0.8722	1.8071	1.5523	1.6024	1.6757	1.7493	2.4717

Key	1	2	3	4	5	6
A	blank	blank	0.0078125*ul*/mL Std.	0.0078125*ul*/mL Std.	MC3-ConA	MC3-ConA
B	1*ul*/mL Std.	1*ul*/mL Std.	MC1-B	MC1-B	MC3-DNP-KLH	MC3-DNP-KLH
C	0.5*ul*/mL Std.	0.5*ul*/mL Std.	MC1-ConA	MC1-ConA	MT1-B	MT1-B
D	0.25*ul*/mL Std.	0.25*ul*/mL Std.	MC1-DNP-KLH	MC1-DNP-KLH	MT1-ConA	MT1-ConA
E	0.125*ul*/mL Std.	0.125*ul*/mL Std.	MC2-B	MC2-B	MT1-DNP-KLH	MT1-DNP-KLH
F	0.0625*ul*/mL Std.	0.0625*ul*/mL Std.	MC2-ConA	MC2-ConA	MT2-B	MT2-B
G	0.03125*ul*/mL Std.	0.03125*ul*/mL Std.	MC2-DNP-KLH	MC2-DNP-KLH	MT2-ConA	MT2-ConA
H	0.015625*ul*/mL Std.	0.015625*ul*/mL Std.	MC3-B	MC3-B	MT2-DNP-KLH	MT2-DNP-KLH

7	8	9	10	11	12
MT3-B	MT3-B	MT5-DNP-KLH	MT5-DNP-KLH	MT8-ConA	MT8-ConA
MT3-ConA	MT3-ConA	MT6-B	MT6-B	MT8-DNP-KLH	MT8-DNP-KLH
MT3-DNP-KLH	MT3-DNP-KLH	MT6-ConA	MT6-ConA	MT9-B	MT9-B
MT4-B	MT4-B	MT6-DNP-KLH	MT6-DNP-KLH	MT9-ConA	MT9-ConA
MT4-ConA	MT4-ConA	MT7-B	MT7-B	MT9-DNP-KLH	MT9-DNP-KLH
MT4-DNP-KLH	MT4-DNP-KLH	MT7-ConA	MT7-ConA		
MT5-B	MT5-B	MT7-DNP-KLH	MT7-DNP-KLH		
MT5-ConA	MT5-ConA	MT8-B	MT8-B		

Mean and Std. Dev. (Male):

Sample	Mean	Std. Dev.
MC-B	2.3872	0.556811674
MC-ConA	2.130616667	0.733529628
MC-DNP-KLH	2.328816667	0.588817443
MT-B	2.585738889	0.443036229
MT-ConA	2.622194444	0.43719642
MT-DNP-KLH	2.693927778	0.656027472

O.D. Values (Female):

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.6782	1.9782	Overflow	2.5802	Overflow	Overflow	2.387	2.6304	Overflow	Overflow	0.0692	0.0332
B	Overflow	Overflow	1.5235	Overflow	0.9659	1.2646	1.2825	2.8763	1.6165	2.2838	0.034	0.0961
C	2.2185	Overflow	2.6562	2.8397	1.2656	0.8724	1.0831	Overflow	Overflow	2.4615	0.032	0.0348
D	1.8546	Overflow	2.3789	0.9172	1.2768	2.6148	1.3011	1.0458	0.7737	1.0217	0.031	0.0313
E	1.3511	Overflow	0.7935	0.8471	2.019	1.3479	2.0518	1.9623	0.2089	0.0945	0.0325	0.0286
F	1.2455	Overflow	1.0579	1.4668	1.5853	1.4515	1.512	1.4851	0.8542	0.1096	0.0344	0.0275
G	1.0593	2.1817	1.4843	1.5455	Overflow	2.6055	Overflow	2.1421	Overflow	0.4796	0.0289	0.0285
H	0.8656	1.7647	1.6076	1.9829	2.4024	1.2628	2.5966	1.0522	0.2292	0.1477	0.0279	0.0344

Key	1	2	3	4	5	6
A	blank	blank	0.0078125*u*l/mL Std.	0.0078125*u*l/mL Std.	FC3-ConA	FC3-ConA
B	1*u*l/mL Std.	1*u*l/mL Std.	FC1-B	FC1-B	FC3-DNP-KLH	FC3-DNP-KLH
C	0.5*u*l/mL Std.	0.5*u*l/mL Std.	FC1-ConA	FC1-ConA	FT1-B	FT1-B
D	0.25*u*l/mL Std.	0.25*u*l/mL Std.	FC1-DNP-KLH	FC1-DNP-KLH	FT1-ConA	FT1-ConA
E	0.125*u*l/mL Std.	0.125*u*l/mL Std.	FC2-B	FC2-B	FT1-DNP-KLH	FT1-DNP-KLH
F	0.0625*u*l/mL Std.	0.0625*u*l/mL Std.	FC2-ConA	FC2-ConA	FT2-B	FT2-B
G	0.03125*u*l/mL Std.	0.03125*u*l/mL Std.	FC2-DNP-KLH	FC2-DNP-KLH	FT2-ConA	FT2-ConA
H	0.015625*u*l/mL Std.	0.015625*u*l/mL Std.	FC3-B	FC3-B	FT2-DNP-KLH	FT2-DNP-KLH

7	8	9	10	11	12
FT3-B	FT3-B	FT5-DNP-KLH	FT5-DNP-KLH		
FT3-ConA	FT3-ConA	FT6-B	FT6-B		
FT3-DNP-KLH	FT3-DNP-KLH	FT6-ConA	FT6-ConA		
FT4-B	FT4-B	FT6-DNP-KLH	FT6-DNP-KLH		
FT4-ConA	FT4-ConA				
FT4-DNP-KLH	FT4-DNP-KLH				
FT5-B	FT5-B				
FT5-ConA	FT5-ConA				

Mean and Std. Dev. (Female):

Sample	Mean	Std. Dev.
FC-B	1.625766667	0.735518848
FC-ConA	2.336766667	0.938967881
FC-DNP-KLH	1.426066667	0.277285919
FT-B	1.798458333	0.652001383
FT-ConA	2.231691667	0.423425086
FT-DNP-KLH	1.825641667	0.694847535

ANOVA

Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	58	18.02	**<.0001**	
TMT	1	58	2.59	0.1127	
Culture conditions	2	58	0.81	0.4478	
TMT=Control					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	16	2.47	0.1352	
TMT=Mercury-exposed					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	43	16.96	**0.0002**	
Media Alone					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	10.34	**0.0048**	
TMT	1	18	0.49	0.4919	
	MA TMT=Control				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	4	2.04	0.226
	MA TMT=Mercury-exposed				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	13	7.85	**0.015**
DNP-KLH					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	10.56	**0.0044**	
TMT	1	18	1.66	0.2141	
	D TMT=Control				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	4	5.77	0.0742

	D TMT=Mercury-exposed				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	13	6.02	**0.029**
ConA					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	0.75	0.3972	
TMT	1	18	0.6	0.4477	

Across culture conditions: Means and Student's *t*-test, two-sample, unequal variance

Unexposed control mean	2.039206
Mercury-exposed mean	2.361144
Unexposed control vs. mercury-exposed	0.095336
Mercury-exposed male mean	2.633953704
Mercury-exposed female mean	1.951930556
Mercury exposed: male vs. female	0.000377247
Unexposed control male mean	2.282211111
Unexposed control female mean	1.7962
Unexposed control : male vs. female	0.136686394

Media Alone: Overall means

Unexposed control mean	2.006483
Mercury-exposed mean	2.270827

ConA: Overall means

Unexposed control mean	2.233692
Mercury-exposed mean	2.465993

DNP-KLH: Overall means

Unexposed control mean	1.877442
Mercury-exposed mean	2.346613

Student's *t*-test:

Paired

Comparison	
MC-B vs. MC-ConA	0.656337795
MC-B vs. MC-DNP-KLH	0.906733883
MT-B vs. MT-ConA	0.862727824
MT-B vs. MT-DNP-KLH	0.687988802

Two-sample, unequal variances

Comparison	
MC-B vs. MT-B	0.61508121
MC-ConA vs. MT-ConA	0.366985
MC-DNP-KLH vs. MT-DNP-KLH	0.41952774

Paired

Comparison	
FC-B vs. FC-ConA	0.363275999
FC-B vs. FC-DNP-KLH	0.694390991
FT-B vs. FT-ConA	0.206964209
FT-B vs. FT-DNP-KLH	0.945672091

Two-sample, unequal variances

Comparison	
FC-B vs. FT-B	0.7493199
FC-ConA vs. FT-ConA	0.86785821
FC-DNP-KLH vs. FT-DNP-KLH	0.25994795

Two-sample, unequal variances

Comparison	
MC-B vs. FC-B	0.23103225
MC-ConA vs. FC-ConA	0.78018434
MC-DNP-KLH vs. FC-DNP-KLH	0.10034206
MT-B vs. FT-B	**0.03205515**
MT-ConA vs. FT-ConA	0.11177123
MT-DNP-KLH vs. FT-DNP-KLH	**0.0349108**

Appendix 4C: Data from ELISA Assay for Cytokines

T_H2:

IL-4 Data:

O.D. Values (Male):

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.6615	1.1582	1.817	1.8415	2.2902	1.7001	1.9262	2.1885	2.0342	2.1071	2.5559	Overflow
B	2.8289	Overflow	2.3489	2.5415	2.372	2.1449	2.4203	2.0277	1.8722	2.3554	1.7467	2.2216
C	1.6139	2.6868	2.244	2.6677	2.1663	2.7993	2.0682	1.7604	2.2081	2.8002	2.0656	1.2528
D	1.1169	1.8034	1.7627	1.7529	2.392	2.6357	2.1784	2.2083	1.7477	1.8848	2.5063	1.4255
E	1.0718	1.73	2.0642	2.6107	1.6647	Overflow	Overflow	Overflow	1.6664	1.5628	1.7325	1.1013
F	0.818	2.0789	1.7575	1.8715	2.2233	1.9471	1.928	1.6803	1.3596	1.5736	1.2407	0.5746
G	0.7493	1.7534	1.1709	2.1359	1.9244	1.6797	1.1391	0.9963	0.8016	2.0077	0.8155	0.5123
H	0.4168	0.7345	1.4402	1.5971	1.1927	0.5983	2.2423	2.1576	0.7478	1.4677	0.3522	0.5331

Key	1	2	3	4	5	6
A	blank	blank	0.00390625*u*l/mL Std.	0.00390625*u*l/mL Std.	MC3-ConA	MC3-ConA
B	0.5*u*l/mL Std.	0.5*u*l/mL Std.	MC1-B	MC1-B	MC3-DNP-KLH	MC3-DNP-KLH
C	0.25*u*l/mL Std.	0.25*u*l/mL Std.	MC1-ConA	MC1-ConA	MT1-B	MT1-B
D	0.125*u*l/mL Std.	0.125*u*l/mL Std.	MC1-DNP-KLH	MC1-DNP-KLH	MT1-ConA	MT1-ConA
E	0.0625*u*l/mL Std.	0.0625*u*l/mL Std.	MC2-B	MC2-B	MT1-DNP-KLH	MT1-DNP-KLH
F	0.03125*u*l/mL Std.	0.03125*u*l/mL Std.	MC2-ConA	MC2-ConA	MT2-B	MT2-B
G	0.015625*u*l/mL Std.	0.015625*u*l/mL Std.	MC2-DNP-KLH	MC2-DNP-KLH	MT2-ConA	MT2-ConA
H	0.0078125*u*l/mL Std.	0.0078125*u*l/mL Std.	MC3-B	MC3-B	MT2-DNP-KLH	MT2-DNP-KLH

7	8	9	10	11	12
MT3-B	MT3-B	MT5-DNP-KLH	MT5-DNP-KLH	MT8-ConA	MT8-ConA
MT3-ConA	MT3-ConA	MT6-B	MT6-B	MT8-DNP-KLH	MT8-DNP-KLH
MT3-DNP-KLH	MT3-DNP-KLH	MT6-ConA	MT6-ConA	MT9-B	MT9-B
MT4-B	MT4-B	MT6-DNP-KLH	MT6-DNP-KLH	MT9-ConA	MT9-ConA
MT4-ConA	MT4-ConA	MT7-B	MT7-B	MT9-DNP-KLH	MT9-DNP-KLH
MT4-DNP-KLH	MT4-DNP-KLH	MT7-ConA	MT7-ConA		
MT5-B	MT5-B	MT7-DNP-KLH	MT7-DNP-KLH		
MT5-ConA	MT5-ConA	MT8-B	MT8-B		

Mean and Std. Dev. (Male):

Sample	Mean	Std. Dev.
MC-B	2.042183333	0.521364293
MC-ConA	2.116233333	0.262327778
MC-DNP-KLH	1.929366667	0.387207726
MT-B	1.820194444	0.491551037
MT-ConA	2.272716667	0.483356028
MT-DNP-KLH	1.737655556	0.431582981

O.D. Values (Female):

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.3049	1.6676	1.2375	1.3082	1.3179	1.1649	1.5956	1.3411	1.2523	1.8155	0.03	0.029
B	2.987	Overflow	1.6827	1.5203	0.9369	1.3987	2.1011	2.0621	1.6489	1.5318	0.0315	0.0328
C	Overflow	Overflow	2.7088	2.7813	1.0542	1.1624	1.3415	1.8953	Overflow	Overflow	0.0338	0.0308
D	2.7463	Overflow	1.3278	1.0071	2.0503	2.0073	1.4072	1.3781	1.1741	1.3263	0.0339	0.0325
E	1.5232	2.3722	1.1423	1.0102	1.1346	1.0035	Overflow	2.6662	0.5893	0.3379	0.0325	0.0308
F	1.2083	1.953	2.1886	2.1365	0.9966	1.0467	1.3408	0.9577	0.3506	0.7033	0.0344	0.0373
G	1.0869	1.387	1.063	1.0777	2.7185	1.8903	1.1964	0.9521	0.4128	0.3054	0.0353	0.0334
H	0.8876	0.9347	0.6559	0.7887	0.79	0.65	1.782	1.7387	0.1947	0.0786	0.0328	0.0338

Key	1	2	3	4	5	6
A	blank	blank	0.00390625*u*l/mL Std.	0.00390625*u*l/mL Std.	FC3-ConA	FC3-ConA
B	0.5*u*l/mL Std.	0.5*u*l/mL Std.	FC1-B	FC1-B	FC3-DNP-KLH	FC3-DNP-KLH
C	0.25*u*l/mL Std.	0.25*u*l/mL Std.	FC1-ConA	FC1-ConA	FT1-B	FT1-B
D	0.125*u*l/mL Std.	0.125*u*l/mL Std.	FC1-DNP-KLH	FC1-DNP-KLH	FT1-ConA	FT1-ConA
E	0.0625*u*l/mL Std.	0.0625*u*l/mL Std.	FC2-B	FC2-B	FT1-DNP-KLH	FT1-DNP-KLH
F	0.03125*u*l/mL Std.	0.03125*u*l/mL Std.	FC2-ConA	FC2-ConA	FT2-B	FT2-B
G	0.015625*u*l/mL Std.	0.015625*u*l/mL Std.	FC2-DNP-KLH	FC2-DNP-KLH	FT2-ConA	FT2-ConA
H	0.0078125*u*l/mL Std.	0.0078125*u*l/mL Std.	FC3-B	FC3-B	FT2-DNP-KLH	FT2-DNP-KLH

7	8	9	10	11	12
FT3-B	FT3-B	FT5-DNP-KLH	FT5-DNP-KLH		
FT3-ConA	FT3-ConA	FT6-B	FT6-B		
FT3-DNP-KLH	FT3-DNP-KLH	FT6-ConA	FT6-ConA		
FT4-B	FT4-B	FT6-DNP-KLH	FT6-DNP-KLH		
FT4-ConA	FT4-ConA				
FT4-DNP-KLH	FT4-DNP-KLH				
FT5-B	FT5-B				
FT5-ConA	FT5-ConA				

Mean and Std. Dev. (Female):

Sample	Mean	Std. Dev.
FC-B	1.13335	0.442372544
FC-ConA	2.049666667	0.758154216
FC-DNP-KLH	1.1352	0.05616202
FT-B	1.275925	0.237883126
FT-ConA	2.334708333	0.485686541
FT-DNP-KLH	1.223466667	0.327379648

ANOVA

Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	53	14.53	**0.0004**	
TMT	1	53	0.13	0.7229	
Culture conditions	2	53	13.33	**<.0001**	
Sex*TMT	1	53	1.14	0.2915	
TMT* **Culture conditions**	2	53	0.57	0.5716	
Sex* Culture conditions	2	53	3.86	**0.0271**	
Sex=Female					
Effect	Num DF	Den DF	F Value	Pr>F	
TMT	1	23	1.14	0.2972	
Culture conditions	2	23	20.36	**<.0001**	
Sex=Male					
Effect	Num DF	Den DF	F Value	Pr>F	
TMT	1	32	0.25	0.6238	
Culture conditions	2	32	3.33	**0.0485**	
TMT=Control					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	16	5.88	**0.0275**	
TMT=Mercury-exposed					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	43	3.79	**0.0580**	
Media Alone					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	11.84	**0.0029**	
TMT	1	18	0.06	0.8107	
	MA TMT=Control				

	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	4	5.3	0.0827
	MA TMT=Mercury-exposed				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	13	6.26	**0.0265**
DNP-KLH					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	13.53	**0.0017**	
TMT	1	18	0.11	0.7397	
	DNP-KLH TMT=Control				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	4	12.36	**0.0245**
	DNP-KLH TMT=Mercury-exposed				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	13	6.11	**0.0281**
ConA					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	0.01	0.9128	
TMT	1	18	0.83	0.3751	

Across culture conditions: Overall means

Unexposed control mean	1.734333
Mercury-exposed mean	1.81066
Mercury-exposed male mean	1.943522
Mercury-exposed female mean	1.611367
Unexposed control male mean	2.029261
Unexposed control female mean	1.439406
Unexposed control : male vs. female	**0.031035**

Media Alone: Overall means

Unexposed control mean	1.587767
Mercury-exposed mean	1.602487

ConA: Overall means

Unexposed control mean	2.08295
Mercury-exposed mean	2.297513

DNP-KLH: Overall Means

Unexposed control mean	1.532283
Mercury-exposed mean	1.53198

Student's *t*-test:

Paired

Comparison	
MC-B vs. MC-ConA	0.840379794
MC-B vs. MC-DNP-KLH	0.77967519
MT-B vs. MT-ConA	0.06649961
MT-B vs. MT-DNP-KLH	0.710085151

Two-sample, unequal variances

Comparison	
MC-B vs. MT-B	0.55955371
MC-ConA vs. MT-ConA	0.50240514
MC-DNP-KLH vs. MT-DNP-KLH	0.51232786

Paired

Comparison	
FC-B vs. FC-ConA	0.16198377
FC-B vs. FC-DNP-KLH	0.994900859
FT-B vs. FT-ConA	**0.001780929**
FT-B vs. FT-DNP-KLH	0.757987565

Two-sample, unequal variances

Comparison	
FC-B vs. FT-B	0.64297927
FC-ConA vs. FT-ConA	0.59673007
FC-DNP-KLH vs. FT-DNP-KLH	0.54653935

Two-sample, unequal variances

Comparison	
MC-B vs. FC-B	0.08448878
MC-ConA vs. FC-ConA	0.89661324
MC-DNP-KLH vs. FC-DNP-KLH	0.06805028
MT-B vs. FT-B	**0.01421419**
MT-ConA vs. FT-ConA	0.8128179
MT-DNP-KLH vs. FT-DNP-KLH	**0.02161878**

Appendix 4D: Data from ELISA Assay for Cytokines

T_H2:

IL-10 Data:

O.D. Values (Male):

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.7105	2.9783	2.2729	1.8761	2.2425	2.4308	1.7692	2.8555	2.7075	2.4046	2.9549	Overflow
B	2.5036	2.8298	Overflow	Overflow	1.9576	2.5052	2.8059	2.6694	2.938	2.8105	1.5691	2.9546
C	2.353	2.1238	Overflow	2.5852	2.3925	2.8162	2.7493	2.804	2.5052	2.7719	2.0671	2.47
D	2.0234	2.1748	2.062	1.362	2.3631	Overflow	2.2436	2.4647	2.2335	2.6722	2.0358	1.8496
E	1.4516	2.0675	1.9657	2.0153	2.2661	2.5413	2.4171	2.2833	2.0486	2.5202	2.4031	1.9287
F	1.2635	1.5074	1.8601	2.6909	1.8177	2.0809	1.93	2.3087	1.7132	1.8123	1.1192	1.0296
G	1.2453	2.1118	2.3472	Overflow	2.2688	2.8238	2.025	2.0505	2.3582	1.9336	1.2065	0.8461
H	0.7766	1.6454	2.0954	1.698	1.6922	1.7565	1.7066	1.8554	1.43	1.629	0.8723	0.4998

Key	1	2	3	4	5	6
A	blank	blank	0.015625*ul*/mL Std.	0.015625*ul*/mL Std.	MC3-ConA	MC3-ConA
B	2*ul*/mL Std.	2*ul*/mL Std.	MC1-B	MC1-B	MC3-DNP-KLH	MC3-DNP-KLH
C	1*ul*/mL Std.	1*ul*/mL Std.	MC1-ConA	MC1-ConA	MT1-B	MT1-B
D	0.5*ul*/mL Std.	0.5*ul*/mL Std.	MC1-DNP-KLH	MC1-DNP-KLH	MT1-ConA	MT1-ConA
E	0.25*ul*/mL Std.	0.25*ul*/mL Std.	MC2-B	MC2-B	MT1-DNP-KLH	MT1-DNP-KLH
F	0.125*ul*/mL Std.	0.125*ul*/mL Std.	MC2-ConA	MC2-ConA	MT2-B	MT2-B
G	0.0625*ul*/mL Std.	0.0625*ul*/mL Std.	MC2-DNP-KLH	MC2-DNP-KLH	MT2-ConA	MT2-ConA
H	0.03125*ul*/mL Std.	0.03125*ul*/mL Std.	MC3-B	MC3-B	MT2-DNP-KLH	MT2-DNP-KLH

7	8	9	10	11	12
MT3-B	MT3-B	MT5-DNP-KLH	MT5-DNP-KLH	MT8-ConA	MT8-ConA
MT3-ConA	MT3-ConA	MT6-B	MT6-B	MT8-DNP-KLH	MT8-DNP-KLH
MT3-DNP-KLH	MT3-DNP-KLH	MT6-ConA	MT6-ConA	MT9-B	MT9-B
MT4-B	MT4-B	MT6-DNP-KLH	MT6-DNP-KLH	MT9-ConA	MT9-ConA
MT4-ConA	MT4-ConA	MT7-B	MT7-B	MT9-DNP-KLH	MT9-DNP-KLH
MT4-DNP-KLH	MT4-DNP-KLH	MT7-ConA	MT7-ConA		
MT5-B	MT5-B	MT7-DNP-KLH	MT7-DNP-KLH		
MT5-ConA	MT5-ConA	MT8-B	MT8-B		

Mean and Std. Dev. (Male):

Sample	Mean	Std. Dev.
MC-B	2.04295	0.074175501
MC-ConA	2.259	0.023334524
MC-DNP-KLH	2.231066667	0.386783729
MT-B	2.246066667	0.385026907
MT-ConA	2.379794444	0.447522867
MT-DNP-KLH	2.289622222	0.302716191

O.D. Values (Female):

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.5	1.473	2.0474	1.5031	1.5372	1.6262	1.429	1.6019	1.7587	2.0687	0.0333	0.0282
B	1.8846	2.1825	1.1433	1.9458	1.512	1.6166	1.715	2.6264	1.7431	1.6167	0.0306	0.0334
C	1.7244	1.7545	1.3054	1.9271	1.6786	1.8886	1.5764	1.8063	1.7468	1.6046	0.0293	0.0279
D	1.7461	2.9658	1.2289	2.189	1.6531	2.4788	1.6515	2.7256	1.6011	1.6484	0.031	0.0314
E	1.7543	1.3915	1.3313	1.6793	1.7754	2.0458	1.8685	1.5869	0.06	0.0566	0.0329	0.0311
F	1.6293	1.9181	1.3649	1.4428	1.7332	2.5996	1.8596	1.6478	0.059	0.0644	0.0367	0.0322
G	1.7377	Overflow	2.5437	2.7995	2.0673	2.3627	2.0211	1.9505	0.2185	0.1897	0.0304	0.0312
H	1.6319	1.7432	2.7428	1.8674	1.4441	2.2414	1.7888	1.7292	0.1499	0.105	0.0302	0.0367

Key	1	2	3	4	5	6
A	blank	blank	0.015625*u*l/mL Std.	0.015625*u*l/mL Std.	FC3-ConA	FC3-ConA
B	2*u*l/mL Std.	2*u*l/mL Std.	FC1-B	FC1-B	FC3-DNP-KLH	FC3-DNP-KLH
C	1*u*l/mL Std.	1*u*l/mL Std.	FC1-ConA	FC1-ConA	FT1-B	FT1-B
D	0.5*u*l/mL Std.	0.5*u*l/mL Std.	FC1-DNP-KLH	FC1-DNP-KLH	FT1-ConA	FT1-ConA
E	0.25*u*l/mL Std.	0.25*u*l/mL Std.	FC2-B	FC2-B	FT1-DNP-KLH	FT1-DNP-KLH
F	0.125*u*l/mL Std.	0.125*u*l/mL Std.	FC2-ConA	FC2-ConA	FT2-B	FT2-B
G	0.0625*u*l/mL Std.	0.0625*u*l/mL Std.	FC2-DNP-KLH	FC2-DNP-KLH	FT2-ConA	FT2-ConA
H	0.03125*u*l/mL Std.	0.03125*u*l/mL Std.	FC3-B	FC3-B	FT2-DNP-KLH	FT2-DNP-KLH

7	8	9	10	11	12
FT3-B	FT3-B	FT5-DNP-KLH	FT5-DNP-KLH		
FT3-ConA	FT3-ConA	FT6-B	FT6-B		
FT3-DNP-KLH	FT3-DNP-KLH	FT6-ConA	FT6-ConA		
FT4-B	FT4-B	FT6-DNP-KLH	FT6-DNP-KLH		
FT4-ConA	FT4-ConA				
FT4-DNP-KLH	FT4-DNP-KLH				
FT5-B	FT5-B				
FT5-ConA	FT5-ConA				

Mean and Std. Dev. (Female):

Sample	Mean	Std. Dev.
FC-B	1.784983333	0.450861565
FC-ConA	1.533933333	0.113972281
FC-DNP-KLH	1.981616667	0.601904192
FT-B	1.886616667	0.272172259
FT-ConA	1.935675	0.241779006
FT-DNP-KLH	1.789475	0.119150354

ANOVA

Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	57	26.83	**<.0001**	
TMT	1	57	0.05	0.8319	
Culture conditions	2	57	0.17	0.8418	
Sex*TMT	1	57	0.68	0.4122	
TMT=Control					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	16	9.06	**0.0083**	
TMT=Mercury-exposed					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	43	19.91	**<0.0001**	
Media Alone					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	6.26	**0.0222**	
TMT	1	18	0.01	0.9426	
	MA TMT=Control				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	4	1.95	0.2348
	MA TMT=Mercury-exposed				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	13	3.89	0.0704
ConA					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	12.77	**0.0022**	
TMT	1	18	0.44	0.5143	

	ConA TMT=Control				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	4	14.44	**0.0191**
	ConA TMT=Mercury-exposed				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	13	4.87	**0.0459**
DNP-KLH					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	8.81	**0.0082**	
TMT	1	18	0.14	0.7101	
	DNP-KLH TMT=Control				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	4	0.36	0.5785
	DNP-KLH TMT=Mercury-exposed				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	13	14.56	**0.0021**

Across culture conditions: Overall means

Unexposed control mean	2.066594
Mercury-exposed mean	2.131332
Mercury-exposed male mean	2.305161111
Mercury-exposed female mean	1.870588889
Mercury exposed: male vs. female	**1.2827E-05**
Unexposed control male mean	2.366344444
Unexposed control female mean	1.766844444
Unexposed control : male vs. female	**0.008294256**

Media alone: Overall means

Unexposed control mean	2.073475
Mercury-exposed mean	2.102287

ConA: Overall means

Unexposed control mean	1.533933
Mercury-exposed mean	2.202147

DNP-KLH: Overall means

Unexposed control mean	2.106342
Mercury-exposed mean	2.089563

Student's *t*-test:

Paired

Comparison	
MC-B vs. MC-ConA	0.740992428
MC-B vs. MC-DNP-KLH	0.756263038
MT-B vs. MT-ConA	0.506770368
MT-B vs. MT-DNP-KLH	0.793228691

Two-sample, unequal vaiances

Comparison	
MC-B vs. MT-B	0.76163055
MC-ConA vs. MT-ConA	0.68692001
MC-DNP-KLH vs. MT-DNP-KLH	0.82717964

Paired

Comparison	
FC-B vs. FC-ConA	0.438749692
FC-B vs. FC-DNP-KLH	0.675868126
FT-B vs. FT-ConA	0.748240936
FT-B vs. FT-DNP-KLH	0.4500912

Two-sample, unequal vaiances

Comparison	
FC-B vs. FT-B	0.74523019
FC-ConA vs. FT-ConA	**0.0116957**
FC-DNP-KLH vs. FT-DNP-KLH	0.63708976

Two-sample, unequal variances

Comparison	
MC-B vs. FC-B	0.23759974
MC-ConA vs. FC-ConA	**0.05083487**
MC-DNP-KLH vs. FC-DNP-KLH	0.58374409
MT-B vs. FT-B	**0.05424077**
MT-ConA vs. FT-ConA	**0.02789708**
MT-DNP-KLH vs. FT-DNP-KLH	**0.00091699**

Appendix 5A: Data from ELISA Assay for DNP-KLH-specific Immunoglobulins

IgM:

O.D. Values (Male):

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.557	0.6115	0.5813	0.6374	0.4928	0.4256	0.755	0.6816	1.116	1.0415	0.035	0.0343
B	0.6725	0.4813	0.423	0.6105	0.5124	0.5225	0.473	0.3092	0.3635	0.3894	0.0354	0.0332
C	0.5737	0.767	0.7987	0.4194	0.4517	0.3457	1.0866	0.7239	0.3402	0.4404	0.0368	0.0323
D	0.805	0.6603	0.4315	0.4272	0.6254	0.5291	0.4842	0.3102	0.5346	0.4927	0.0357	0.0347
E	0.4992	0.5923	0.2993	0.3172	1.3997	1.0507	0.3015	0.2102	0.2398	0.3357	0.0352	0.0343
F	0.8705	0.5183	0.4323	0.3946	0.3693	0.3611	0.2936	0.2463	0.3132	0.3198	0.0383	0.0232
G	0.379	0.4139	0.6852	0.5112	0.5975	0.5457	0.2681	0.1815	0.2347	0.4144	0.0329	0.0329
H	0.6296	0.3678	0.2768	0.2902	1.8638	2.5998	0.3727	0.2894	0.8799	0.303	0.0256	0.0328

Key	1	2	3	4	5	6
A	blank	blank	MC3-LPS	MC3-LPS	MT3-B	MT3-B
B	MC1-B	MC1-B	MC3-DNP-KLH	MC3-DNP-KLH	MT3-LPS	MT3-LPS
C	MC1-LPS	MC1-LPS	MT1-B	MT1-B	MT3-DNP-KLH	MT3-DNP-KLH
D	MC1-DNP-KLH	MC1-DNP-KLH	MT1-LPS	MT1-LPS	MT4-B	MT4-B
E	MC2-B	MC2-B	MT1-DNP-KLH	MT1-DNP-KLH	MT4-LPS	MT4-LPS
F	MC2-LPS	MC2-LPS	MT2-B	MT2-B	MT4-DNP-KLH	MT4-DNP-KLH
G	MC2-DNP-KLH	MC2-DNP-KLH	MT2-LPS	MT2-LPS	MT5-B	MT5-B
H	MC3-B	MC3-B	MT2-DNP-KLH	MT2-DNP-KLH	MT5-LPS	MT5-LPS

7	8	9	10	11	12
MT5-DNP-KLH	MT5-DNP-KLH	MT8-LPS	MT8-LPS		
MT6-B	MT6-B	MT8-DNP-KLH	MT8-DNP-KLH		
MT6-LPS	MT6-LPS	MT9-B	MT9-B		
MT6-DNP-KLH	MT6-DNP-KLH	MT9-LPS	MT9-LPS		
MT7-B	MT7-B	MT9-DNP-KLH	MT9-DNP-KLH		
MT7-LPS	MT7-LPS				
MT7-DNP-KLH	MT7-DNP-KLH				
MT8-B	MT8-B				

Mean and Std. Dev. (Male):

Sample	Mean	Std. Dev.
MC-B	0.54045	0.03936848
MC-LPS	0.65803333	0.04384234
MC-DNP-KLH	0.54861667	0.1703503
MT-B	0.44431667	0.12056248
MT-LPS	0.86328889	0.602543
MT-DNP-KLH	0.37335	0.14229524

O.D. Values (Female):

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.3586	0.338	0.3075	0.2759	0.2812	0.3132	0.2592	0.3285	0.0317	0.0308	0.0332	0.0349
B	0.4514	0.3842	0.3544	0.3839	0.407	0.5889	0.3494	0.6334	0.0341	0.0363	0.0337	0.0324
C	0.4582	1.0287	0.3293	0.3257	Overflow	0.4382	1.204	1.9956	0.0344	0.0442	0.0324	0.0335
D	0.3931	0.36	0.6039	0.4159	0.4206	0.5504	0.7026	0.3824	0.0344	0.0337	0.0338	0.0337
E	0.5549	0.7621	0.4426	0.3691	0.8031	1.1427	0.2907	2.897	0.0339	0.0342	0.0338	0.0355
F	1.0361	1.0602	0.3047	0.3529	0.3168	0.2855	0.3809	0.3966	0.0358	0.0331	0.0342	0.0373
G	0.497	0.354	0.61	0.704	0.4064	0.3787	0.3262	0.365	0.0346	0.0364	0.037	0.0316
H	0.4592	0.3821	0.3782	0.5508	0.5391	0.5682	0.2797	0.5394	0.0235	0.0256	0.0238	0.0274

Key	1	2	3	4	5	6
A	blank	blank	FC3-LPS	FC3-LPS	FT3-B	FT3-B
B	FC1-B	FC1-B	FC3-DNP-KLH	FC3-DNP-KLH	FT3-LPS	FT3-LPS
C	FC1-LPS	FC1-LPS	FT1-B	FT1-B	FT3-DNP-KLH	FT3-DNP-KLH
D	FC1-DNP-KLH	FC1-DNP-KLH	FT1-LPS	FT1-LPS	FT4-B	FT4-B
E	FC2-B	FC2-B	FT1-DNP-KLH	FT1-DNP-KLH	FT4-LPS	FT4-LPS
F	FC2-LPLS	FC2-LPS	FT2-B	FT2-B	FT4-DNP-KLH	FT4-DNP-KLH
G	FC2-DNP-KLH	FC2-DNP-KLH	FT2-LPS	FT2-LPS	FT5-B	FT5-B
H	FC3-B	FC3-B	FT2-DNP-KLH	FT2-DNP-KLH	FT5-LPS	FT5-LPS

7	8	9	10	11	12
FT5-DNP-KLH	FT5-DNP-KLH				
FT6-B	FT6-B				
FT6-LPS	FT6-LPS				
FT6-DNP-KLH	FT6-DNP-KLH				

Mean and Std. Dev. (Female):

Sample	Mean	Std. Dev.
FC-B	0.498983333	0.138152835
FC-LPS	0.694433333	0.380599692
FC-DNP-KLH	0.3904	0.030621847
FT-B	0.387158333	0.084408284
FT-LPS	0.798533333	0.430369859
FT-DNP-KLH	0.407675	0.096587446

ANOVA

Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	57	0.26	0.6102	
TMT	1	57	0.01	0.9083	
Culture conditions	2	57	10.9	**<.0001**	
Sex*TMT	1	57	0.02	0.8776	
Media Alone					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	1.31	0.2674	
TMT	1	18	4.24	**0.0543**	
	MA Sex=Female				
	Effect	Num DF	Den DF	F Value	Pr>F
	TMT	1	7	2.37	0.1674
	MA Sex=Male				
	Effect	Num DF	Den DF	F Value	Pr>F
	TMT	1	10	1.74	0.2163
DNP-KLH					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	0.03	0.8708	
TMT	1	18	0.46	0.5063	
LPS					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	0.15	0.7046	
TMT	1	18	1.78	0.1986	

Across culture conditions: Overall means

Unexposed control mean	0.555153
Mercury-exposed mean	0.54864

LPS: Overall means

Unexposed control mean	0.676233
Mercury-exposed mean	0.837387

DNP-KLH: Overall means

Unexposed control mean	0.469508
Mercury-exposed mean	0.38708

Student's *t*-test:

Paired

Comparison	
MC-B vs. MC-LPS	**0.018697081**
MC-B vs. MC-DNP-KLH	0.934666217
MT-B vs. MT-LPS	0.054888391
MT-B vs. MT-DNP-KLH	0.1533966

Two-sample, unequal variances

Comparison	
MC-B vs. MT-B	0.06427375
MC-LPS vs. MT-LPS	0.339435561
MC-DNP-KLH vs. MT-DNP-KLH	0.206871844

Paired

Comparison	
FC-B vs. FC-LPS	0.353865936
FC-B vs. FC-DNP-KLH	0.223365996
FT-B vs. FT-LPS	**0.03900527**
FT-B vs. FT-DNP-KLH	0.720747755

Two-sample, unequal variances

Comparison	
FC-B vs. FT-B	0.29486252
FC-LPS vs. FT-LPS	0.727727873
FC-DNP-KLH vs. FT-DNP-KLH	0.702047021

Two-sample, unequal variances

Comparison	
MC-B vs. FC-B	0.660504652
MC-LPS vs. FC-LPS	0.884069072
MC-DNP-KLH vs. FC-DNP-KLH	0.246748947
MT-B vs. FT-B	0.300020504
MT-LPS vs. FT-LPS	0.812088869
MT-DNP-KLH vs. FT-DNP-KLH	0.587353136

Appendix 5B: Data from ELISA Assay for DNP-KLH-specific Immunoglobulins

IgE:

O.D. Values (Male):

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.3802	0.6104	0.2403	0.2179	0.2138	0.2486	0.2535	0.271	0.3552	0.2301	0.0466	0.0355
B	0.3824	0.339	0.3498	0.2434	0.2475	0.2654	0.2544	0.7802	0.2356	0.2488	0.0368	0.0354
C	1.411	0.2704	0.2244	0.2764	0.2253	0.2339	0.235	0.2276	0.5814	0.1955	0.0365	0.0389
D	0.2999	0.5549	0.3002	0.2383	0.2553	0.239	0.2216	0.2191	0.5617	0.2271	0.0378	0.0399
E	0.2858	0.2633	0.3099	0.2442	0.2851	0.2807	0.2178	0.259	0.2265	0.2512	0.0453	0.0402
F	0.3434	0.2688	0.256	0.21	0.2437	0.2416	0.3633	0.2329	0.2963	0.2946	0.0395	0.036
G	0.2972	0.2225	0.2514	0.2435	0.3382	0.2265	0.2112	0.231	0.228	0.2639	0.045	0.0377
H	0.3937	0.2535	0.299	0.2883	0.3057	0.3168	0.3205	0.2559	0.249	0.2691	0.0286	0.0428

Key	1	2	3	4	5	6
A	blank	blank	MC3-LPS	MC3-LPS	MT3-B	MT3-B
B	MC1-B	MC1-B	MC3-DNP-KLH	MC3-DNP-KLH	MT3-LPS	MT3-LPS
C	MC1-LPS	MC1-LPS	MT1-B	MT1-B	MT3-DNP-KLH	MT3-DNP-KLH
D	MC1-DNP-KLH	MC1-DNP-KLH	MT1-LPS	MT1-LPS	MT4-B	MT4-B
E	MC2-B	MC2-B	MT1-DNP-KLH	MT1-DNP-KLH	MT4-LPS	MT4-LPS
F	MC2-LPS	MC2-LPS	MT2-B	MT2-B	MT4-DNP-KLH	MT4-DNP-KLH
G	MC2-DNP-KLH	MC2-DNP-KLH	MT2-LPS	MT2-LPS	MT5-B	MT5-B
H	MC3-B	MC3-B	MT2-DNP-KLH	MT2-DNP-KLH	MT5-LPS	MT5-LPS

7	8	9	10	11	12
MT5-DNP-KLH	MT5-DNP-KLH	MT8-LPS	MT8-LPS		
MT6-B	MT6-B	MT8-DNP-KLH	MT8-DNP-KLH		
MT6-LPS	MT6-LPS	MT9-B	MT9-B		
MT6-DNP-KLH	MT6-DNP-KLH	MT9-LPS	MT9-LPS		
MT7-B	MT7-B	MT9-DNP-KLH	MT9-DNP-KLH		
MT7-LPS	MT7-LPS				
MT7-DNP-KLH	MT7-DNP-KLH				
MT8-B	MT8-B				

Mean and Std. Dev. (Male):

Sample	Mean	Std. Dev.
MC-B	0.319616667	0.043212913
MC-LPS	0.458633333	0.333111773
MC-DNP-KLH	0.32795	0.088064564
MT-B	0.297383333	0.095983146
MT-LPS	0.287083333	0.047709747
MT-DNP-KLH	0.247522222	0.025252501

O.D. Values (Female):

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.3252	0.3339	0.2562	0.2862	0.3295	0.3461	0.3246	0.573	0.0406	0.0397	0.0347	0.036
B	0.3443	0.2492	0.3611	0.2437	0.2394	0.3058	0.3862	0.2802	0.0413	0.0358	0.0351	0.0366
C	1.6186	0.4429	0.3992	0.3382	0.4916	0.2992	0.2908	0.2949	0.0385	0.0401	0.0396	0.0381
D	0.2531	0.2218	0.8995	0.8491	0.3188	0.2734	0.3324	0.2736	0.0369	0.0414	0.0386	0.0377
E	0.4828	0.2555	0.2422	0.4497	0.5686	0.2691	0.2768	0.2414	0.0358	0.0382	0.0377	0.0454
F	0.3346	0.3064	0.4517	0.4535	0.5487	0.289	0.2887	0.2978	0.0347	0.0374	0.0425	0.0384
G	0.5643	0.3117	0.3423	0.3309	0.3029	0.3257	0.2768	0.3261	0.0318	0.0294	0.0391	0.0308
H	0.3882	0.343	0.2793	0.3228	0.2888	0.2853	0.4538	0.2916	0.0457	0.0394	0.0411	0.0291

Key	1	2	3	4	5	6
A	blank	blank	FC3-LPS	FC3-LPS	FT3-B	FT3-B
B	FC1-B	FC1-B	FC3-DNP-KLH	FC3-DNP-KLH	FT3-LPS	FT3-LPS
C	FC1-LPS	FC1-LPS	FT1-B	FT1-B	FT3-DNP-KLH	FT3-DNP-KLH
D	FC1-DNP-KLH	FC1-DNP-KLH	FT1-LPS	FT1-LPS	FT4-B	FT4-B
E	FC2-B	FC2-B	FT1-DNP-KLH	FT1-DNP-KLH	FT4-LPS	FT4-LPS
F	FC2-LPLS	FC2-LPS	FT2-B	FT2-B	FT4-DNP-KLH	FT4-DNP-KLH
G	FC2-DNP-KLH	FC2-DNP-KLH	FT2-LPS	FT2-LPS	FT5-B	FT5-B
H	FC3-B	FC3-B	FT2-DNP-KLH	FT2-DNP-KLH	FT5-LPS	FT5-LPS

7	8	9	10	11	12
FT5-DNP-KLH	FT5-DNP-KLH				
FT6-B	FT6-B				
FT6-LPS	FT6-LPS				
FT6-DNP-KLH	FT6-DNP-KLH				

Mean and Std. Dev (Female):

Sample	Mean	Std. Dev.
FC-B	0.343833333	0.040813978
FC-LPS	0.540816667	0.425010148
FC-DNP-KLH	0.32595	0.102328039
FT-B	0.35045	0.05565252
FT-LPS	0.413708333	0.231809166
FT-DNP-KLH	0.368841667	0.061732288

ANOVA

Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	57	3	0.0886	
TMT	1	57	2.26	0.1382	
Culture conditions	2	57	1.88	0.1621	
Sex*TMT	1	57	0.71	0.4042	
Media Alone					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	1.88	0.1874	
TMT	1	18	0.06	0.8108	
LPS					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	1.36	0.2592	
TMT	1	18	1.99	0.1754	
DNP-KLH					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	17	4.23	0.0554	
TMT	1	17	0.42	0.5264	
Sex*TMT	1	17	4.52	**0.0485**	
	DNP-KLH TMT=Control				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	4	0	0.9808
	DNP-KLH TMT=Mercury-exposed				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	13	28.52	**0.0001**
	DNP-KLH Sex=Female				
	Effect	Num DF	Den DF	F Value	Pr>F

	TMT	1	7	0.64	0.4487
	DNP-KLH Sex=Male				
	Effect	Num DF	Den DF	F Value	Pr>F
	TMT	1	10	7.06	**0.024**

Across culture conditions: Overall means

Unexposed control mean	0.386133
Mercury-exposed mean	0.317464

Media alone: overall means

Unexposed control mean	0.331725
Mercury-exposed mean	0.31861

LPS: Overall means

Unexposed control mean	0.499725
Mercury-exposed mean	0.337733

Student's *t*-test:

Paired

Comparison	
MC-B vs. MC-LPS	0.508834976
MC-B vs. MC-DNP-KLH	0.803471873
MT-B vs. MT-LPS	0.775507204
MT-B vs. MT-DNP-KLH	0.207995135

Two-sample, unequal variances

Comparison	
MC-B vs. MT-B	0.598044119
MC-LPS vs. MT-LPS	0.466724581
MC-DNP-KLH vs. MT-DNP-KLH	0.252596949

Paired

Comparison	
FC-B vs. FC-LPS	0.539960344
FC-B vs. FC-DNP-KLH	0.720151877
FT-B vs. FT-LPS	0.532064836
FT-B vs. FT-DNP-KLH	0.695160758

Two-sample, unequal variances

Comparison	
FC-B vs. FT-B	0.847031634
FC-LPS vs. FT-LPS	0.666393161
FC-DNP-KLH vs. FT-DNP-KLH	0.555880645

Two-sample, unequal variances

Comparison	
MC-B vs. FC-B	0.519447084
MC-LPS vs. FC-LPS	0.805811819
MC-DNP-KLH vs. FC-DNP-KLH	0.980784293
MT-B vs. FT-B	0.199558136
MT-LPS vs. FT-LPS	0.241278341
MT-DNP-KLH vs. FT-DNP-KLH	**0.00362526**

Appendix 5C: Data from ELISA Assay for DNP-KLH-specific Immunoglobulins

IgG:

O.D. Values (Male):

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.7053	1.6739	1.7786	1.496	1.4164	1.6387	1.3649	1.7301	1.7811	0.9977	0.0334	0.0274
B	1.5116	1.1651	1.6428	1.3535	1.6343	1.3173	1.7274	1.8163	1.3049	1.0118	0.0313	0.0327
C	1.6729	1.6767	1.6576	1.6925	0.922	1.0349	1.5701	1.4664	1.7506	1.7281	0.0387	0.0291
D	1.5819	1.4212	1.9987	1.8659	1.7311	1.4173	1.1507	1.3143	1.8764	1.4915	0.0293	0.0329
E	1.1581	1.354	1.1373	1.2715	1.4668	1.4062	1.6076	1.1906	1.4739	1.3952	0.0403	0.0443
F	1.0199	2.4963	1.4927	1.3493	1.4131	1.2748	1.5488	1.3169	1.4099	1.3291	0.0396	0.0252
G	1.349	0.9883	1.4135	1.2926	1.2901	1.3963	1.4915	1.267	1.4694	1.3318	0.0371	0.0288
H	1.0013	1.4225	1.1889	1.2603	1.5928	1.1416	1.4114	1.1684	0.744	1.179	0.032	0.0271

Key	1	2	3	4	5	6
A	blank	blank	MC3-LPS	MC3-LPS	MT3-B	MT3-B
B	MC1-B	MC1-B	MC3-DNP-KLH	MC3-DNP-KLH	MT3-LPS	MT3-LPS
C	MC1-LPS	MC1-LPS	MT1-B	MT1-B	MT3-DNP-KLH	MT3-DNP-KLH
D	MC1-DNP-KLH	MC1-DNP-KLH	MT1-LPS	MT1-LPS	MT4-B	MT4-B
E	MC2-B	MC2-B	MT1-DNP-KLH	MT1-DNP-KLH	MT4-LPS	MT4-LPS
F	MC2-LPS	MC2-LPS	MT2-B	MT2-B	MT4-DNP-KLH	MT4-DNP-KLH
G	MC2-DNP-KLH	MC2-DNP-KLH	MT2-LPS	MT2-LPS	MT5-B	MT5-B
H	MC3-B	MC3-B	MT2-DNP-KLH	MT2-DNP-KLH	MT5-LPS	MT5-LPS

7	8	9	10	11	12
MT5-DNP-KLH	MT5-DNP-KLH	MT8-LPS	MT8-LPS		
MT6-B	MT6-B	MT8-DNP-KLH	MT8-DNP-KLH		
MT6-LPS	MT6-LPS	MT9-B	MT9-B		
MT6-DNP-KLH	MT6-DNP-KLH	MT9-LPS	MT9-LPS		
MT7-B	MT7-B	MT9-DNP-KLH	MT9-DNP-KLH		
MT7-LPS	MT7-LPS				
MT7-DNP-KLH	MT7-DNP-KLH				
MT8-B	MT8-B				

Mean and Std. Dev. (Male):

Sample	Mean	Std. Dev.
MC-B	1.26876667	0.06417699
MC-LPS	1.69006667	0.06183012
MC-DNP-KLH	1.38945	0.19122597
MT-B	1.5268	0.17563595
MT-LPS	1.50992222	0.18733573
MT-DNP-KLH	1.27817222	0.16812345

O.D. Values (Female):

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.1502	1.1227	1.0426	1.2853	1.1482	1.1049	1.1052	1.1383	0.0348	0.038	0.0382	0.038
B	1.2029	1.0607	1.2906	1.4284	1.2055	1.1398	1.5736	1.6978	0.0322	0.0358	0.0564	0.0363
C	0.6734	1.0912	1.3284	1.2663	1.3389	1.1866	1.3176	1.3609	0.0379	0.0372	0.0404	0.0565
D	1.4152	1.2084	1.4206	1.2994	1.2762	0.9742	1.1447	1.3272	0.039	0.0371	0.0567	0.052
E	0.9684	1.5421	1.0565	1.265	1.237	1.4896	0.2071	0.149	0.0373	0.0453	0.0391	0.0399
F	1.4863	1.6447	1.1117	1.4752	1.4154	1.1601	0.1805	0.1827	0.0399	0.0436	0.0368	0.0407
G	1.2102	1.4775	1.3468	1.3236	1.5085	1.0573	0.1603	0.1533	0.034	0.0368	0.0363	0.0392
H	1.241	1.3873	1.4179	1.4323	1.1265	1.202	0.1439	0.1493	0.031	0.037	0.0371	0.0371

Key	1	2	3	4	5	6
A	blank	blank	FC3-LPS	FC3-LPS	FT3-B	FT3-B
B	FC1-B	FC1-B	FC3-DNP-KLH	FC3-DNP-KLH	FT3-LPS	FT3-LPS
C	FC1-LPS	FC1-LPS	FT1-B	FT1-B	FT3-DNP-KLH	FT3-DNP-KLH
D	FC1-DNP-KLH	FC1-DNP-KLH	FT1-LPS	FT1-LPS	FT4-B	FT4-B
E	FC2-B	FC2-B	FT1-DNP-KLH	FT1-DNP-KLH	FT4-LPS	FT4-LPS
F	FC2-LPLS	FC2-LPS	FT2-B	FT2-B	FT4-DNP-KLH	FT4-DNP-KLH
G	FC2-DNP-KLH	FC2-DNP-KLH	FT2-LPS	FT2-LPS	FT5-B	FT5-B
H	FC3-B	FC3-B	FT2-DNP-KLH	FT2-DNP-KLH	FT5-LPS	FT5-LPS

7	8	9	10	11	12
FT5-DNP-KLH	FT5-DNP-KLH				
FT6-B	FT6-B				
FT6-LPS	FT6-LPS				
FT6-DNP-KLH	FT6-DNP-KLH				

Mean and Std. Dev. (Female):

Sample	Mean	Std. Dev.
FC-B	1.23373333	0.09305969
FC-LPS	1.20391667	0.34334904
FC-DNP-KLH	1.33838333	0.02431534
FT-B	1.293525	0.18623921
FT-LPS	1.28910833	0.09414968
FT-DNP-KLH	1.24900833	0.10663279

ANOVA

Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	53	14.03	**0.0004**	
TMT	1	53	0.01	0.9374	
Culture conditions	2	53	1.92	0.1569	
Sex*TMT	1	53	0.1	0.7534	
TMT* Culture conditions	2	53	3.06	0.0552	
Sex* Culture conditions	2	53	3.16	**0.0507**	
Sex=Female					
Effect	Num DF	Den DF	F Value	Pr>F	
TMT	1	23	0.08	0.7745	
Culture conditions	2	23	0.03	0.9687	
Sex=Male					
Effect	Num DF	Den DF	F Value	Pr>F	
TMT	1	30	0.03	0.8642	
Culture conditions	2	30	6.19	**0.0056**	
TMT* Culture conditions	2	30	4.45	0.0203	
TMT=Control					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	16	4.00	0.0629	
TMT=Mercury-exposed					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	43	8.71	**0.0051**	
Media Alone					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	5.27	**0.0274**	
TMT	1	18	4.26	0.0537	

	MA TMT=Control				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	4	0.29	0.6199
	MA TMT=Mercury-exposed				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	13	6.06	**0.0286**
	MA Sex=Female				
	Effect	Num DF	Den DF	F Value	Pr>F
	TMT	1	7	0.26	0.6242
	MA Sex=Male				
	Effect	Num DF	Den DF	F Value	Pr>F
	TMT	1	10	5.87	**0.0358**
LPS					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	12.75	**0.0022**	
TMT	1	18	0.36	0.5541	
	LPS TMT=Control				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	4	5.83	0.0733
	LPS TMT=Mercury-exposed				
	Effect	Num DF	Den DF	F Value	Pr>F
	Sex	1	13	7.02	**0.02**
DNP-KLH					
Effect	Num DF	Den DF	F Value	Pr>F	
Sex	1	18	0.33	0.5755	
TMT	1	18	2.18	0.1571	

Across culture conditions: Overall means

Unexposed control mean	1.354053
Mercury-exposed mean	1.373864
Mercury-exposed male mean	1.4382981
Mercury-exposed female mean	1.2772139
Mercury exposed: male vs. female	0.002388428
Unexposed control male mean	1.449427778
Unexposed control female mean	1.258677778

LPS: Overall means

Unexposed control mean	1.446992
Mercury-exposed mean	1.421597

DNP-KLH: Overall means

Unexposed control mean	1.363917
Mercury-exposed mean	1.266507

Student's *t*-test:

Paired

Comparison	
MC-B vs. MC-LPS	**0.012654624**
MC-B vs. MC-DNP-KLH	0.386827993
MT-B vs. MT-LPS	0.7364553
MT-B vs. MT-DNP-KLH	**0.017626075**

Two-sample, unequal variances

Comparison	
MC-B vs. MT-B	**0.004267858**
MC-LPS vs. MT-LPS	**0.031493835**
MC-DNP-KLH vs. MT-DNP-KLH	0.432849873

Paired

Comparison	
FC-B vs. FC-LPS	0.878632602
FC-B vs. FC-DNP-KLH	0.118756579
FT-B vs. FT-LPS	0.954998127
FT-B vs. FT-DNP-KLH	0.649502163

Two-sample, unequal variances

Comparison	
FC-B vs. FT-B	0.541393698
FC-LPS vs. FT-LPS	0.711541874
FC-DNP-KLH vs. FT-DNP-KLH	0.099054225

Two-sample, unequal variances

Comparison	
MC-B vs. FC-B	0.623257812
MC-LPS vs. FC-LPS	0.129560296
MC-DNP-KLH vs. FC-DNP-KLH	0.690135076
MT-B vs. FT-B	**0.03452313**
MT-LPS vs. FT-LPS	**0.010514304**
MT-DNP-KLH vs. FT-DNP-KLH	0.687786765