FETAL ORIGIN OF CHRONIC IMMUNE DISEASES:
ROLE OF PRENATAL STRESS CHALLENGE

By

CAITLIN JAGO, B.ARTS.SCI. Hon.

A Thesis Submitted to the School of Graduate Studies
In Partial Fulfillment of the Requirements
For the Degree
Master of Science

McMaster University
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Descriptive Note

Master of Science
(Medical Sciences, Infection and Immunity)  McMaster University

Hamilton, Ontario


AUTHOR: Caitlin Jago (Bachelor of Arts and Science, Hon.)

SUPERVISOR: Dr. Petra Arck

NUMBER OF PAGES: viii, 52
Introduction: Increasing incidence of chronic immune diseases are mirrored by changing disease risk factors, which include maternal stress during pregnancy. To date, no studies have investigated the impact of prenatal stress challenge (PNS) on the fetal immune system. Fetal liver and bone marrow represent major sources of hematopoietic stem cells (HSC) at mid gestation, which differentiate and mature in the thymus. Disturbance of immune development may cause immune impairment in later life. Further, progesterone is recognized as a critical part of feto-maternal interaction. This study aimed to determine if PNS interferes with normal fetal immune development in mice and the impact of progesterone supplementation on stress effects.

Methods: DBA/2J-mated BALB/c dams were sorted into three groups: control, PNS (gestation days (GDs) 12.5 and 14.5) and PNS plus progesterone supplementation (DHD). Fetal tissue was collected on GDs 16.5 and 18.5. Flow cytometric analysis examined frequency and phenotype of fetal immune cell populations: HSC in fetal liver and bone marrow, and different stages of T cell maturation and regulatory T (Treg) cells in the thymus. Fetal tails were collected to determine fetal sex by PCR analysis.

Results: PNS induced a decrease in organ size on GD16.5, which was not seen on GD18.5 and was reversed by DHD treatment. PNS altered the percentage and absolute number of HSC within the liver and bone marrow populations, on GD16.5 and 18.5. There was a significant lag in T cell maturation as demonstrated by the altered expression of CD3 and skewed CD3⁻:CD3⁺ ratio. There was a significant decrease in Treg cells within CD3⁺ thymic cells in response to PNS. PNS effects in the thymus were ameliorated by DHD treatment. There was no PNS-induced sex bias.

Conclusions: These results indicate that PNS compromises the developing fetal immune system, which could account for impaired immune responses in adults with chronic immune disease, and provide evidence for a therapeutic role of progesterone supplementation.
Acknowledgements

I would like to thank my supervisor, Petra Arck, for her supervision, guidance and support over the last years. I am grateful for the many opportunities that you have provided me, personally and professionally.

To my colleagues, both in Canada and Germany, thank you for your support, understanding and humour. In particular, thank you to Emilia, Chris and Isabel for insightful discussion, reassurance and laughter.

To my committee, thank you for asking tough questions and expecting the right answers.

To my friends and family, thank you for listening and supporting me.
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Introduction

Population-based studies have revealed an increase in chronic immune diseases such as allergies, diabetes and multiple sclerosis in industrialized countries over the past six decades\(^1\). This is increasingly being attributed to changing environmental factors, given that the time frame is believed to be too short to allow for genome modifications. Rather, an interaction between genes and the environment is proposed to explain this relatively rapidly occurring development\(^2,3\) particularly in industrialized countries. Epigenetics appear to play a critical role in these interactions, as modifications to the epigenome\(^4\) have been linked to the development of immune diseases such as asthma\(^5,6\) and multiple sclerosis\(^7\). Concurrently, environmental factors such as advances in improved hygiene and socioeconomic status are linked to decreasing infection rates. Interestingly, epidemiological data indicate that the decrease in infections mirrors the increase in incidence of chronic immune disease\(^1\).

In addition, studies have revealed that there are “windows of vulnerability” in which specific environmental factors acting at particular time points in gestation exert specific effects on fetal development\(^8-11\). This research was initially concentrated on the postnatal period, but more recently the prenatal period has been identified as highly susceptible to environmental influences. This concept is referred to as “fetal programming”.

Fetal Programming

Barker first proposed the concept of “fetal programming” in 1989 based on evidence that low birth weight was associated with increased risk for cardiovascular disease in later life\(^12\). The evidence that the prenatal period is critical for not only fetal health but also childhood and adult health is strengthened by further epidemiological and basic science studies investigating the effect of environmental factors such as exposure to pollution\(^13,14\), smoking\(^15-18\), alcohol\(^17,19,20\) and maternal nutrition\(^21-23\) on fetal development. Basic principles of fetal programming state that the effects of programming are permanent, and represent adaptation of physiological systems to unfavourable environmental conditions. The stepwise nature of fetal development means that each phase of development is dependent on the previous stage, and any interference in this coordinated process leads to postnatal consequences\(^24\). The list of environmental programming factors is rapidly expanding, and has been shown to influence the development of humans\(^25,26\), mice\(^11\), and numerous other species\(^27\). Within the last decade this list has been updated to include psychosocial effects on women during pregnancy.

Stress Perception

The existence of a non-specific, broadly occurring stress response was discovered in the early 1930s by Hans Selye\(^28\), who proposed that a “general adaptation” occurred when animals were exposed to noxious stimuli. This
“general adaptation syndrome” became known as “stress”, and has been thoroughly investigated as a potential factor in disease development\textsuperscript{29}. It is a phenomenon that is an initially useful response to environmental stimuli that is harmful if over-activated or inappropriately stimulated\textsuperscript{29}. Further research supported Selye’s initial assertion that stress could affect immune organs such as the thymus, causing involution\textsuperscript{30,31}, and altering hematopoietic tissues in rat fetal liver\textsuperscript{32}.

Allergy was proposed as a stress-linked disease as early as the 1950s\textsuperscript{33-35}, and was added to a diverse list of other immune related conditions such as diabetes mellitus\textsuperscript{36}, dermatological problems\textsuperscript{37}, Graves’ disease\textsuperscript{38} and cardiovascular disease\textsuperscript{39-41}. By the 1970s it was recognized by the medical community as a contributing factor to illnesses, as indicated by a WHO symposium entitled “Society, stress and disease”\textsuperscript{42}. Thus, psychosocial stress became an established environmental factor contributing to chronic disease.

\textit{Prenatal Stress}

In the context of changing environmental factors, the role of women within industrialized society has also changed, as more women are pursuing advanced educational and employment opportunities. Correspondingly, women are also reporting increased stress perception and a higher age at first pregnancy (Fig. 1)\textsuperscript{43}. Thus maternal stress challenges such as anxiety, psychosocial stress and depression during pregnancy are well recognized as risk factors for altered fetal programming in humans, mice, piglets, rats and various other species\textsuperscript{44-47}.

![Figure 1. Changing environmental and lifestyle factors to which women are exposed in industrialized countries during pregnancy.](image)

A variety of epidemiological studies indicate that \textit{in utero} exposure to prenatal stress results in increased risk for cardiovascular disease\textsuperscript{48}, diabetes\textsuperscript{49}, obesity\textsuperscript{23}, asthma\textsuperscript{50,51}, psychological illnesses such as schizophrenia\textsuperscript{52}, and other poor health outcomes including modulation of the fetal neurodevelopment\textsuperscript{44,46,50,53-55}. This is supported by basic science studies, where the effects of prenatal stress challenge have been demonstrated in a variety of experimental settings\textsuperscript{45,56}. As such, it has been proposed that
prenatal stress challenge is a major candidate mechanism for fetal programming\textsuperscript{57}. Within the prenatal period there are critical windows of vulnerability to adverse environmental factors that affect different aspects of fetal development. In 2006, Pincus \textit{et al} developed a robust model of prenatal stress that provided substantial evidence that prenatal sound stress challenge in mid- to late gestation increased the severity of asthma in adult offspring\textsuperscript{58}. Furthermore they identified a skew towards a Th2 biased immune system as demonstrated by an altered adaptive immune response\textsuperscript{58-60}, which indicated a compromise in fetal immune development. This altered immune development represents a potential foundation for the development of a wide range of later life chronic diseases, as fetal programming effects are permanent. As such it could act as a critical intermediate measure of fetal health and later life outcomes. In addition, the effects of fetal programming often demonstrate a sex bias, where either male or female offspring will be preferentially affected by prenatal environmental challenges\textsuperscript{61-65}. While the effects of fetal programming on adult offspring have been well established, few studies have directly examined the impact of \textit{in utero} challenges on the developing fetus.

\textbf{Immune System Development}

It is well established that fetal immune system development in mammals begins prenatally. In 1964, Silverstein proposed that the development of the immune system occurs as a stepwise process throughout gestation\textsuperscript{66}. Numerous discoveries in the intervening decades have outlined these specific stages, and it is now well established that this complex process encompasses fetal immune organ development\textsuperscript{57,68}, hematopoietic stem cell seeding and migration\textsuperscript{69-71}, and the differentiation and maturation of innate and adaptive immune cells\textsuperscript{72-74}. There are numerous similarities between murine and human immune development\textsuperscript{75,76}, which allowed extrapolation and comparison between experimental models and clinical studies. Given that there are particular species-specific differences, such as length of gestation and timing of organogenesis, the following is a description of murine fetal immune development.

\textbf{Hematopoietic Stem Cells}

Hematopoietic stem cells (HSC) are characterized by their capacity for self-renewal and multilineage \textit{in vivo} reconstitution potential. This population acts as a constant supply of progenitor cells for the lymphoid and myeloid lineages. HSC are generally defined\textsuperscript{77-79} as Lin$^{-}$Sca-1$^{+}$c-kit$^{+}$CD34$^{+}$.

The murine fetal liver is the major hematopoietic stem cell source throughout mid- to late-gestation, from gestation day (GD) 11.5 to GD15.5\textsuperscript{71}. It contains a rapidly proliferating population of long term reconstituting multilineage progenitors that peaks at GD14.5 and decreases rapidly after GD15.5\textsuperscript{80}. During this time, the number of fetal liver cells doubles each day while the frequency of HSC remains constant, evidence of continuous HSC expansion in the fetal liver\textsuperscript{70,80}.

Around GD15.5 the fetal bone marrow and, to a lesser extent, the fetal spleen become the major hematopoietic organs\textsuperscript{71}. This occurs concurrently
with a drop in fetal liver HSC frequency and reconstitution capacity\textsuperscript{70,80}. The bone marrow remains hematopoietic throughout adult life\textsuperscript{81}. The concurrent decline in fetal liver HSC frequency and the appearance of HSC in the spleen and bone marrow provides further evidence that the HSC population can emigrate from the hematopoietic organs and seed other tissues, such as the thymus\textsuperscript{80}.

It has been established that there are two waves of HSC that colonize the fetal thymus during gestation. In mice, the first T cell progenitor seeding begins on GD10.5\textsuperscript{67,82} and is thought to primarily originate from the fetal liver\textsuperscript{83}. The second wave occurs between GD12.5 and 16.5\textsuperscript{67}, and displays a high level of T-cell progenitor potential\textsuperscript{67}. Once they have entered the thymus, progenitors proliferate and differentiate before being subjected to extensive selection processes\textsuperscript{82}.

**Figure 2.** Simplified outline of murine fetal immune ontogeny. *Upper part:* Hematopoietic stem cell (HSC) locations in fetal mice. *Lower part:* Liver-derived T cell progenitors (Pro T) seed the thymus, and progress through maturation stages characterized by CD44 and CD25 (DN1-DN4), then by changing expression of CD4, CD8 and CD3.

**Thymic Lymphocytes**

The thymic organ has a unique and specialized role in the development of the fetal immune system. Interactions of thymocyte progenitors with the
thymic stroma and epithelium are central to establishing and maintaining the T cell component of the immune system. Progenitor cells seeding from the hematopoietic organs require these interactions to undergo the correct differentiation and selection processes required to generate functional, mature, self-MHC-restricted T cells. Thus the thymus is critical for the maturation of pre-thymic stem cells to mature T cells.

The triple negative population (TN, CD3⁻CD4⁻CD8⁻) is characterized by expression of CD44 and CD25. These four stages are known as “double negative” (DN) and represent the earliest stages of maturation. DN1 and DN2 represent the pro-thymocyte (pro-T) stage of maturation, DN3 and DN4 represent the pre-thymocyte (pre-T) stage of maturation. Pro-thymocytes are the most immature, and at the DN1 stage are more like common lymphoid progenitors in that they are able to differentiate into T, B or NK cells. As these cells transition through progressive stages of maturation, they become exclusively committed to the T cell lineage. During the DN3 stage, cells undergo T cell receptor (TCR) rearrangements and branch into the gamma-delta or alpha-beta T cell lineages. By the DN4 stage, these cells are referred to as pre-T cells. Downregulation of CD44 and CD25, combined with changing expression of CD4, CD8 and CD3 represent the final five stages of maturation, including positive and negative expression. Prior to selection, there is an intermediate stage represented by intermediate single positive (ISP) thymocytes, characterized as CD3⁺CD4⁺CD8⁻CD44⁻CD25⁻. Upregulation of CD4 signals the transition from ISP to pre-selection double positive (pre-DP) characterized as CD3⁺CD4⁺CD8⁻CD44⁺CD25⁺. As thymocytes undergo positive selection, they upregulate expression of CD3 and become post-selection double positive thymocytes (post-DP), characterized as CD3⁺CD4⁺CD8⁻CD44⁺CD25⁺. The final stage, negative selection, results in single positive (SP) T cells, expressing either CD4 or CD8.

During selection, apoptosis is a vital part of the selection process and occurs primarily between GD15.5 and 18.5 in mice. This process has vital functions: elimination of T cells that respond to self-antigen, elimination of non-functional T cells, and migration of T cells that recognize foreign antigen to the periphery. Given that T cells are long lived, challenges to the maturation and selection process may have long lasting effects for postnatal health.

Of particular interest from the emerging T cell subsets are the regulatory T (Treg) cells. They are identified as CD4⁺CD25⁺ and associated with expression of forkhead/winged-helix family transcriptional repressor p3 (Foxp3). These cells have been shown to have a crucial role in regulating immune responses in adults, mediating homeostasis and tolerance. More recently, Tregs have been identified in fetuses from both humans and mice, although their role requires further clarification. The longevity of Treg cells combined with their critical function in immune homeostasis indicates that challenges through fetal programming could have serious repercussions.

The T cell selection process is highly controlled, and regulated both temporally and spatially within the thymus. Migration through the cortex and into the medulla is accompanied by T cell receptor (TCR) gene rearrangement.
and changes in expression of cell surface markers. Once selection is finalized, various T cell populations seed the periphery in different waves, based on the gestational time point. Disturbance of this carefully orchestrated immune development may be a pivotal cause for immune impairment in later life. Interestingly, certain maternal endocrine biomarkers have been identified as causative for adverse pregnancy outcomes including altering fetal immune development. This includes suppression of maternal progesterone levels, shown in a number of species including humans \(^94,95\), and mice \(^59,96\).

**Role of maternal endocrine function**

Basic science studies have implicated maternal progesterone levels as a mechanism for altered feto-maternal interactions, including altered placental development and subsequent impact on the fetus \(^59,97\). This is supported in clinical studies, which have demonstrated that low levels of maternal progesterone are inversely correlated with increased risk for allergies in female children \(^64\). Thus there is increasing evidence that prenatal stress acts as a potent programming factor by modulating various pregnancy and developmental biomarkers, such as maternal progesterone levels. This increases the risk for offspring to develop impaired immune responses in later life by influencing fetal immune ontogeny *in utero*.

Further research is urgently required to determine the mechanisms by which fetal development is compromised.

**Primary Hypothesis:**
Maternal stress during gestation has immediate consequences for fetal immune development.

**Secondary Hypothesis:**
Stress-triggered alterations in fetal immune development can be reversed through progesterone supplementation of the dam during pregnancy.

**Objectives:**
The following objectives were outlined for this Master’s thesis:

1. Identify if stress has an influence on the development of murine hematopoietic stem cells and the related immune organs (liver, bone marrow) on GD16.5 and GD18.5
   - *Effect of stress on pregnancy outcomes (implantations, abortion rate, male to female ratio) on GD16.5*
   - *Effect of stress on fetal liver on GD16.5*
   - *Effect of stress on pregnancy outcomes on GD18.5*
   - *Effect of stress on fetal liver on GD18.5*
   - *Effect of stress on fetal bone marrow on GD18.5*
2. Identify if stress has an influence on T cell development and thymic organogenesis on GD16.5 and GD18.5
   - *Effect of stress on fetal thymus on GD16.5*
   - *Effect of stress on fetal thymus on GD18.5*
3. Investigate therapeutic effects of progesterone supplementation
a. Effect of stress plus progesterone supplementation on pregnancy outcomes on GD16.5
b. Effect of stress plus progesterone supplementation on fetal liver on GD16.5
c. Effect of stress plus progesterone supplementation on fetal thymus on GD16.5.
Materials and Methods

Mice
6-8 week old virgin female BALB/c mice were obtained from Harlan (Indianapolis, IN) in Canada and from Charles River (Sukzfeld, Germany), and maintained in an animal facility on a 12h light/dark cycle. The mice were given mouse chow and water ad libitum. Mice were allowed to adjust to the facility for one week prior to mating. In Canada, animal care and experimental procedures were conducted according to institutional and McMaster University Animal Research Ethics Board guidelines, and conformed to standards of the Canadian Council of Animal Care. In Germany, work was conducted according to Universitätsklinikum-Hamburg Eppendorf policies, conforming to ethical standards according to the German Animal Welfare Act (Deutsches Tierschutzgesetz).

Maternal Stress Protocol and Experimental Setup
BALB/c females were mated with in-house DBA/2J males. Successful mating was assumed upon the appearance of a vaginal plug, which was designated GD0.5. Dams were also weighed on GD 8.5 and 11.5 to assess pregnancy success. Dams were separated into three groups – control, stress challenge and stress challenge plus dihydrodydrogesterone supplementation (stress plus DHD). Controls were left undisturbed throughout pregnancy. The stress challenge and stress plus DHD group was exposed to sound stress of 75dB at 300Hz emitted for 1 second, 4 times per minute by a device (Conrad Electronic) placed in the mouse cage for 24h on G12.5 and 14.5.

Dihydrodydrogesterone Supplementation
A synthetic progesterone derivative called dihydrodydrogesterone was administered according to previously established experimental design. Each mouse received 1.25mg of DHD dissolved in 200μL of oil solution (20% Benzylboatt and 80% Rizinusoil). This was administered via subcutaneous injection on GD11.5, 13.5 and 15.5, as per established protocols (Fig. 3).

Figure 3. Experimental setup for progesterone supplementation of dams during pregnancy.
Fetal Tissue Collection

Fetuses were selected based on the following inclusion criteria:
1. Litter size between 5 and 12 fetuses
2. Abortion rate lower than 30%
3. Fetal weight between 300mg and 600mg (GD16.5)

Isolation of Thymocytes, Liver Cells and Bone Marrow Cells

Dams were sacrificed on GD16.5 and 18.5 via CO₂ asphyxiation and the uterus was removed. Each feto-placental unit was carefully removed from the uterus and placed in a petri dish filled with PBS, on ice. Fetal distribution in the uterus was documented. The fetus was separated from the placenta. Placental tissue was stored in various conditions for future use. Fetuses were weighed (GD16.5 only) and subsequently tails were removed. Fetal thymus, tail, liver, and serum were collected on GD16.5; fetal thymus, tail, liver, and bone marrow were collected on GD18.5. Fetal tissues were removed using the aid of a stereo microscope and placed into individual Eppendorf tubes containing complete RPMI (cRPMI; 10% FBS, 1% L-Glut, 1% Pen-Strep) on ice.

Tissue was processed to obtain single cell suspensions in cRPMI. RBC Lysis Buffer (eBiosciences) was used to purify fetal liver and bone marrow (500µL for 10 minutes at room temperature, washed with 20mL ice cold PBS, spun for 3 minutes at 300g). Cells were resuspended in cRPMI, and 10µL aliquots were removed. These were stained with Trypan blue and viable cells counted using a hemocytometer prior to fluorescence-activated cell sorting (FACS) analysis. On GD16.5, thymus cells were counted at a dilution of 1:10 (10µL of cells, 90µL of Trypan blue), and liver and bone marrow cells were counted at a dilution of 1:100 (10µL of cells, 900µL of Trypan blue, 900µL of cRPMI). Absolute cell numbers in fetal thymus, liver and bone marrow were documented. FACS analysis was used to further exclude potential dead cells and other debris based on granularity and size.

Fetal blood was obtained and spun for 20 minutes at 10000 x g. Supernatant containing the serum was separated and stored at -20°C.

Antibodies and Flow Cytometry

Isolated fetal cells were added to a 96-well plate. On GD16.5, 3x10⁵ thymus cells and 1x10⁶ liver cells were added to each well; on GD18.5, 1x10⁶ thymus, liver or bone marrow cells were added to each well. Cells were spun for 3 minutes at 300g, then resuspended in 200µL FACS buffer (PBS with 0.25% BSA). Cells were spun again for 3 minutes at 300g.

A 50µL solution of FcBlock (BD Biosciences) at a 1:450 dilution was added to each well to prevent non-specific binding, and cells were incubated on ice for 15 minutes. Cells were washed with 150µL of FACS buffer, and spun for 3 minutes at 300g. 50µL solutions of antibodies against the respective surface antigens were added at optimized dilutions, and cells were incubated on ice for 30 minutes. Cells were washed with 150µL of FACS buffer, and cells were spun for 3 minutes at 300g. Cells were then permeabilized for 20 minutes using 100µL of CytoFix/CytoPerm (BD
Biosciences), and washed with 100µL of PermWash (BD Biosciences). Cells were spun for 3 minutes at 300g. 50µL solutions of antibodies against intracellular antigens were added as required, and cells were incubated on ice for 30 minutes. In wells where no intracellular staining was required, 50µL of PermWash was added. After incubation cells were washed with 150µL of PermWash and spun for 3 minutes at 300g. All cells were then fixed by adding 200µL of BD Stabilizing Fixative (BD Biosciences) to each well, and acquired. Acquisition was performed in Canada using FACSCanto (BD) and in Germany using FACSCalibur (BD), with compensation set using single-colour stained samples. Data was analyzed using FloJo software (FloJo, TreeStar) to investigate frequency and phenotype of fetal immune cell populations. Liver-derived HSC were identified as lineage (Lin)$^-$, CD34$^+$, c-kit (CD117)$^+$, Sca-1$^+$. T cell maturation stages were identified by the presence or absence of CD4, CD8, CD3, CD44 and CD25. Regulatory T cells were identified as CD3$^+$CD4$^+$CD25$^{+++}$Foxp3$^+$. FMOs were used to confirm the negative and positive populations.

DN1 (ProT):  CD3$^-$CD4$^-$CD8$^-$CD44$^{+++}$CD25$^-$
DN2 (ProT):  CD3$^-$CD4$^-$CD8$^-$CD44$^{+++}$CD25$^{++}$
DN3 (PreT):  CD3$^-$CD4$^-$CD8$^-$CD44$^+$CD25$^{++}$
DN4 (PreT):  CD3$^-$CD4$^-$CD8$^-$CD44$^{lo}$CD25$^{lo}$
ISP:            CD3$^-$CD4$^-$CD8$^+$CD44$^-$CD25$^-$
Pre-DP:        CD3$^-$CD4$^+$CD8$^-$CD44$^{CD25}$
Post-DP:       CD3$^-$CD4$^+$CD8$^-$CD44 CD25$^-$
SP CD4:        CD3$^+$CD4$^+$CD8$^-$CD44 CD25$^-$
SP CD8:        CD3$^+$CD4$^+$CD8$^+$CD44 CD25$^-$
Treg:          CD3$^+$CD4$^+$CD25$^{+++}$Foxp3$^+$

**Antibody optimization**

Optimization was performed to titer the antibodies and obtain the correct combination using previously established flow cytometry protocols. Initial titrations were performed for each antibody-colour combination at 1:50, 1:100, 1:200, 1:400 and 1:800. After the initial trial was completed, dot plots were analyzed to determine optimal dilution (Fig. 4-6). This was based on identifying an optimal separation of negative and positive populations. Subsequent titrations were performed at 1:1600 for combinations that were highly expressed. All FACS experiments were performed utilizing this information.
After titration, optimal antibody concentrations were determined as:

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</tr>
<tr>
<td>APC-Cy7</td>
<td>CD8 1:100</td>
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</tr>
<tr>
<td>PE CD44</td>
<td>1:400</td>
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</tr>
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**Figure 4.** Representative dot plots for optimal antibody concentrations for fetal thymus FACS staining.

**Figure 5.** Representative dot plots for optimal antibody concentrations for fetal liver FACS staining.
PCR\textsuperscript{98} for Fetal Sex Determination

Fetal tails were collected on GDs 16.5 and 18.5 for PCR analysis to determine fetal gender based on the presence or absence of the Y chromosome. DNA isolation was completed using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Canada, Mississauga, Canada). Polymerase Chain Reaction (PCR) was employed to detect the presence or absence of the Y chromosome according to established protocol\textsuperscript{98} to elucidate a sex-sensitivity with regard to changes of the immune system. The 402 bp band (Sry) obtained identifies the male fetuses and the 544 bp product (IL3) confirms the correct amplification of the template DNA (Fig. 7).

![Figure 6. Representative dot plots for optimal antibody concentrations for fetal bone marrow FACS staining.](image)

![Figure 7. PCR amplification of fetal DNA. Lanes 1-6 represent female and male DNA amplified with both IL3 and Sry primers. Size of the PCR products are indicated on the side of the figure. Presence or absence of the Y chromosome was used to determine fetal sex.](image)
Thymic Sections

Fetuses from BALB/c x DBA/2J matings were harvested on GD16.5 and 18.5. Fetal weight was obtained, and tails were collected for sex determination. Fetuses were submerged in Bouin’s solution for 48-72h, then placed in 70% ethanol. The ethanol was changed every 48h for one week, then left undisturbed. After approximately two to three weeks, fetuses were removed and thymic sections were obtained. Using “The Atlas of Mouse Development” as a reference, two cuts were made to isolate the thymic section – at the base of the ribcage and the base of the jaw. These sections were placed in cassettes and again submerged in 70% ethanol. The remainder of the fetus was discarded.

Tissues were embedded in paraffin and sectioned. A 5µm section was obtained every 30µm, and stained with H&E. Approximately 15 sections were obtained from each fetus. Analysis was conducted using Zeiss AxioVision software (Zeiss, Germany). Using the “The Atlas of Mouse Development” as a guide, sections were analyzed from the superior to inferior aspects of the thymus and measurements of total thymic area were obtained for each section.

Statistical Analysis

Statistical analysis was conducted using SPSS StatPack for Graduate Students (SPSS, IMB Inc.). The independent Student’s T-test was used with Levine’s F test to check for variance. This was confirmed with a Wilcoxon-Mann-Whitney non-parametric test whenever possible.
Results

Gestation Day 16.5

Prenatal stress challenge had no effect on pregnancy outcomes on GD16.5.

Assessment of pregnancy data on GD16.5 revealed that maternal stress challenge resulted in no significant differences in the implantation (Fig. 8A) or abortion rates (Fig. 8B) for each pregnancy. There were also no significant differences in sex distribution as measured by the male to female offspring ratio, although the proportion of females was increased in response to stress challenge.

Evaluating overall cell number, a decrease in absolute liver cells could be observed in prenatally stressed fetuses compared to controls, which was significant in female offspring (Fig. 9A). While the total percentage of HSC among GD16.5 liver cells was higher in prenatally stressed fetuses compared to controls (Fig. 9B), the absolute number of HSC in liver cells was the same in control and prenatally stressed fetuses (Fig. 9C). HSC were gated on total liver cells as Lineage-⁺, CD34⁺⁻, Sca-1⁺ and c-kit⁺ (Fig. 9D).

Figure 8. Prenatal stress challenge has no effect on pregnancy outcomes on GD16.5. There was no effect of stress exposure on (A) average implantations (B) abortion rate, or (D) the fetal male to female ratio. (C) Representative photo of implantations in the uterus. The results represent the mean ± SE for each sex group. (Student’s Independent T-test).

Maternal stress challenge resulted in changes in fetal liver on GD16.5.

Evaluating overall cell number, a decrease in absolute liver cells could be observed in prenatally stressed fetuses compared to controls, which was significant in female offspring (Fig. 9A). While the total percentage of HSC among GD16.5 liver cells was higher in prenatally stressed fetuses compared to controls (Fig. 9B), the absolute number of HSC in liver cells was the same in control and prenatally stressed fetuses (Fig. 9C). HSC were gated on total liver cells as Lineage⁻⁺, CD34⁻⁻, Sca-1⁺ and c-kit⁺ (Fig. 9D).
Maternal stress challenge induced changes in fetal thymus on GD16.5.

Analysis of the GD16.5 thymus revealed that there were no significant changes in the overall thymic area (Fig. 10A), determined by comparison of thymic histology (Fig. 10B). There were also no significant changes in overall thymic volume (Fig. 10C) which was determined by MRI imaging (Fig. 10D). There was a decrease in total thymic cells (Fig. 10E) in response to stress challenge, which was significant in male offspring. Prenatally stress challenged offspring also had a significantly decreased percentage of regulatory T cells within the CD3⁺ population when compared to controls (Fig. 10F) in male and female offspring.

Figure 9. Maternal stress resulted in changes in fetal liver development on GD16.5. Comparison of stress and control within sex groups. Fetal livers were harvested on GD16.5. Cells were isolated and stained for hematopoietic stem cells (HSC). HSC defined as Lineage CD34⁻ c-kit⁻ Sca-1⁻. (A) Prenatal stress induced a decrease in absolute number of liver cells which was significant in males (P<0.05) and females (p<0.01). (B) Percentage of HSC were increased within total liver cells in response to stress challenge. (C) Absolute number of HSC within liver cells did not change in response to stress challenge. (D) Representative dot plots of HSC in control and stress fetuses, gated on total liver cells. The results represent the mean ± SE for each sex group (*P < 0.05, **P < 0.01 as compared with control). (Student’s Independent T-test).

Maternal stress challenge induced changes in fetal thymus on GD16.5.
Figure 10. Stress challenge induced changes in fetal thymic development on GD16.5. Comparison of stress and control within sex groups. Fetal thymuses were harvested on GD16.5. Single cell suspensions were stained for CD3, CD4, CD8, CD44, CD25 and Foxp3. (A) Mean thymic area (µm²) did not change in response to stress challenge. (B) Representative histology of fetal thymus from control and stressed offspring. (C) Mean thymic volume (mm³) did not change in response to stress challenge. (D) Representative MRI image photo of control fetus on GD16.5, lines indicate location of thymus and liver. (E) Absolute number of thymic cells was decreased in response to stress challenge, significantly in male offspring (p<0.05). (F) Percentage of regulatory T cells (Treg) within the CD3⁺ thymocyte population was significantly decreased in response to stress challenge in both male and female offspring (p<0.05). Treg defined as CD3⁺CD4⁺CD25+++Foxp3⁺. (G) Developmental ratio based on CD3 surface marker expression. Thymocyte populations in male and female offspring were significantly predisposed to a less mature, CD3⁻ phenotype in response to stress challenge (males p<0.05; females p<0.01). Pre-selection double positive (CD25 CD44 CD3 CD8⁻CD4⁺) compared to post-selection double positive (CD25 CD44 CD3⁺CD8⁺CD4⁻) fetal thymic T cells. (H) Representative dot plots of pre-selection and post-selection double positive populations and Treg populations, gated on total thymic cells. The results represent the mean ± SE for each sex group (*P < 0.05 as compared with control, **P < 0.01 as compared with control) (Student’s Independent T-test).
Upregulation of CD3 is an important step during T cell development, and is a strong indicator of T cell maturity\textsuperscript{85,86}. Comparing pre- and post-selection populations by a ratio of the frequency of CD3\textsuperscript{−} to CD3\textsuperscript{+} cells in the thymus served as a reliable measure of fetal maturation status. The ratio of pre-selection (CD3\textsuperscript{−}CD8\textsuperscript{−}CD4\textsuperscript{+}) to post-selection (CD3\textsuperscript{+}CD8\textsuperscript{−}CD4\textsuperscript{+}) double positive thymic cells was significantly skewed towards the less mature pre-selection double positive population in response to stress challenge (Fig. 10G) in male and female offspring. SP CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells were not included in this analysis due to the low proportion of cells in these subsets, which indicated that they would not contribute to any difference in maturation. Prenatal stress challenge produced no significant differences between prenatally stressed and control offspring at the DN1 to DN2 stages (Fig. 11A, B). It did result in a significantly higher percentage of DN3 and DN4 cells in the prenatally stressed thymocyte population when compared to controls in both male and female offspring (Fig. 11A, B). Stress challenged offspring had a significantly higher percentage of ISP in both male and female offspring (Fig. 11C) and a higher percentage of pre-DP thymocytes compared to controls, significant in male offspring (Fig. 11D). They displayed a lower percentage of post-DP thymocytes compared to controls (Fig. 11E), which was significant in female offspring. There were no significant differences following stress challenge in relation to SP CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells (Fig. 12A, B).
Figure 11. Prenatal stress has an effect on developing T cell populations within the fetal thymus. Comparison of stress and control on the percentage of DN cells within the total thymocyte population on GD16.5 for male (A) and female (B) offspring. There was an overall trend towards a higher percentage of DN cell populations in stress challenged offspring. The DN3 and DN4 population was significantly higher on GD16.5 in both male and female offspring in response to stress challenge (p<0.05). The results represent the mean ± SE for each sex group (*P < 0.05 as compared with control). (Student’s Independent T-test). (C) Prenatal stress resulted in significantly higher populations of ISP thymocytes on GD16.5 (p<0.01). (D) There was a higher percentage of pre-DP cells in response to prenatal stress challenge on GD16.5, significantly in males (p<0.05). (E) Control offspring had a higher percentage of post-DP cells compared to prenatally stressed offspring on GD16.5. This supports the alteration in T cell maturation shown by the skewed CD3⁺:CD3⁻ ratio. ISP defined as CD3⁻CD44⁺CD25⁻CD25⁺CD8⁺, pre-DP defined as CD3⁻CD44⁺CD25⁻CD4⁺CD8⁺, and post-DP defined as CD3⁺CD44⁺CD25⁻CD4⁺CD8⁺. The results represent the mean ± SE for each sex group (*P < 0.05 as compared with control). (Student’s Independent T-test).
Gestation Day 18.5

The purpose of assessing the second time point was to compare the effect of stress at two time points in late gestation (GDs 16.5 and 18.5) in order to analyze the kinetics of fetal programming effects of prenatal stress challenge.

Prenatal stress had no effect on pregnancy outcomes on GD18.5.

Stress challenge resulted in no significant differences in implantation rate (Fig. 13A) or abortion rate (Fig. 13B) when analyzed on GD18.5. There were also no differences in sex distribution (Fig. 13C), although there was a slightly higher proportion of female offspring in the stress challenged litters.

Figure 12. Stress challenges alters the percentage of CD4 and CD8 single positive cells within the total thymocyte population on GD16.5. (A) Prenatal stress results in an altered percentage of CD4 SP in male offspring, but not in female offspring. (B) Prenatal stress resulted in a higher number of CD8 SP cells in both male and female offspring. CD4 SP defined as CD3+CD4+CD25-CD4+CD8-, CD8 SP defined as CD3+CD4+CD25+CD4+CD8+. The results represent the mean ± SE for each sex group. (Student’s Independent T-test).
Stress challenge resulted in minor effects on fetal liver on GD18.5.

Analysis of the liver on GD18.5 revealed that there were no significant differences in absolute liver cell number in response to stress challenge (Fig. 14A). There were no significant differences in the overall liver cell number in stressed male offspring and a slight increase in overall liver cell number in stressed female offspring (Fig. 14A). The decrease in the percentage of HSC (Fig. 14B) and the absolute number of HSC in the fetal liver in response to stress challenge (Fig. 14C) was not significant. The total cell number in the liver remained approximately the same from GD16.5 to GD18.5. Both the percentage and the absolute number of HSC decreased in the fetal liver from GD16.5 to GD18.5.

**Figure 13.** Stress challenge has no effect on pregnancy outcomes on GD18.5. There was no effect of stress exposure on (A) average implantations, (B) abortion rate, or (C) the fetal male to female ratio. The results represent the mean ± SE for each sex group (*P < 0.05 as compared with control). (Student’s Independent T-test).
Stress challenge altered fetal bone marrow on GD18.5.

Evaluation of the bone marrow on GD18.5 was performed because this organ becomes the major source of HSC in late gestation\(^6\), and was excluded from GD16.5 analysis due to technical limitations. Analysis revealed that there was no significant decrease in overall bone marrow cells in response to stress challenge in male and female offspring (Fig. 15A). The percentage of HSC was decreased in response to stress, significantly in female offspring (Fig. 15B), while the absolute number of HSC in bone marrow cells was decreased, significantly in male offspring (Fig. 15C).
Stress challenge had a slight effect on fetal thymus on GD18.5.

On GD18.5, there was no decrease in total thymic cells in response to stress (Fig. 16A) and no significant changes in total thymic area (Fig. 16B), which is demonstrated by comparative histological analysis (Fig. 16C). Stress challenge resulted in a significant decrease in the percentage of regulatory T cells within CD3+ thymic cells, in both male and female offspring (Fig. 16D).

There was a significantly higher percentage of DN2 cells in prenatally stressed females compared to controls (Fig. 17A). A trend appeared towards a higher percentage in DN3 subsets in stressed animals compared to controls, in both male and female offspring (Fig. 17A, B). Pre- and post-selection subsets show no significant changes in response to stress challenge in comparison to control offspring (ISP and pre-DP, Fig. 16C, D; SP CD4+, Fig. 18A), except for a significantly increased percentage of post-DP in female offspring and SP CD8+ in both male and female offspring (post-DP, Fig. 17E; SP CD8+, Fig. 18B).
Figure 16. Stress challenge had a slight effect on fetal thymic development on GD18.5. Comparison of stress and control within sex groups. Fetal thymuses were harvested on GD18.5. Single cell suspensions were stained for CD3, CD4, CD8, CD44, CD25 and Foxp3 and analyzed using FloJo software. (A) Absolute number of thymic cells was not substantially altered in response to stress challenge. (B) Mean thymic area (µm²) was not significantly different in response to stress challenge. (C) Representative histology of fetal thymuses from control and stressed offspring on GD18.5. (D) Stress challenge resulted in a significant decrease in both male and female offspring in the percentage of regulatory T cells (Treg) within the CD3+ thymocyte population. (Treg defined as CD3+CD4+CD25+++ Foxp3+). (E) Representative dot plots of pre-DP and post-DP populations and regulatory T cell populations, gated on total thymic cells. The results represent the mean ± SE for each sex group (*P < 0.05 as compared with control) (Student’s Independent T-test).
Figure 17. Prenatal stress has an effect on developing T cell populations within the fetal thymus. Comparison of stress and control on the percentage of DN cells within the total thymocyte population on GD18.5 for male (A) and female (B) offspring. There was an overall trend towards a higher percentage of DN cell populations in stress challenged offspring. On GD18.5, the DN2 population was significantly higher, but only in female offspring (p<0.05). The results represent the mean ± SE for each sex group (*P < 0.05 as compared with control). (Student’s Independent T-test). (C) Prenatal stress did not alter ISP populations. (D) There was no change in the percentage of pre-DP cells in response to prenatal stress challenge, but there was a lower percentage in female offspring compared to males. (E) On GD18.5, prenatal stress resulted in a significantly higher percentage of post-DP cells in female offspring (p<0.05). This supports the alteration in T cell maturation shown by the skewed CD3⁺:CD3⁻ ratio. ISP defined as CD3⁻CD44⁻CD25⁻CD4⁺CD8⁺, pre-DP defined as CD3⁻CD44⁻CD25⁺CD4⁺CD8⁺, and post-DP defined as CD3⁺CD44⁺CD25⁺CD4⁺CD8⁺. The results represent the mean ± SE for each sex group (*P < 0.05 as compared with control). (Student’s Independent T-test). 

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The absolute number of thymic cells increased between GD16.5 and GD18.5. On GD16.5, control animals had a higher proportion of phenotypically mature T cells (as defined by upregulation of CD3) than the stressed offspring. On GD18.5, fetal T cells populations appeared similar in stress offspring compared to controls with the exception of the post-DP subset in females and the CD8+ SP subset in both male and female offspring. Interestingly, prenatally stressed offspring has a significantly higher proportion of cells in these subsets as compared to controls (Fig. 17E, 18B).

Analysis of the overall thymocyte populations demonstrated that the DN subsets are predominant on GD16.5 (Fig. 12A, B and Fig. 17A, B). On GD18.5, the pre-DP subset represents the largest proportion of overall thymocytes (Fig. 17D). A distribution of the analyzed T cells subsets by group (control or stress challenged) is shown for GD16.5 and 18.5 (Appendix: Fig. A1B and A1C, respectively). The percentage of regulatory T cells in the CD3+ T cell population was also higher on GD18.5 compared to GD16.5, in both control and stressed offspring (Fig. 10F and Fig. 16D).

**Progesterone Supplementation**

The second aim of this thesis was to analyze the effect of synthetic progesterone supplementation (dihydroxydronosterone, DHD) on the effects of prenatal stress challenge. The stress-induced decrease of maternal
progesterone levels during pregnancy has been documented by our group in both plasma⁹⁶ (Fig. 19A) and urine (Fig. 19B) (ME Solano, unpublished data).

Stress challenge and stress plus DHD resulted in no changes in pregnancy outcome on GD16.5.

There were no differences in implantation rate between controls, stress and DHD-treated animals (Fig. 18A). The abortion rate was highest in control animals when compared to stress and DHD-treated animals, but not significantly increased (Fig. 18B). Analysis of sex distribution within litters revealed that controls had a lower proportion of males, stressed animals had a higher proportion of males, and the ratio between males and females was almost the same in DHD-treated animals (Fig. 18D). There was a decrease in fetal weight in response to stress challenge that was further decreased in response to DHD supplementation (Fig. 18E).
Stress challenge produced an effect on fetal liver on GD16.5 that was ameliorated by DHD supplementation.

The effects of stress challenge seen in the first experiments were reproduced when the progesterone group was included for evaluation. Analyzing liver cell numbers on GD16.5 revealed that there was an increase in response to DHD treatment, which was significant in males between stress and DHD treatment (Fig. 21A). The percentage of HSC within total liver cells was significantly decreased in female offspring in prenatally stressed compared to control animals (Fig. 21B). The absolute number of HSC was also decreased in prenatally stressed animals compared to controls, significantly in female offspring (Fig. 21C), and increased in response to DHD supplementation (Fig. 21C).

**Figure 20.** Effect of stress and stress plus DHD supplementation on pregnancy outcomes on GD16.5. There was no effect of stress exposure or stress plus DHD treatment on (A) average implantations, (B) abortion rate, or (D) the fetal male to female ratio. (C) Representative photos of implantations from control, stress challenge and stress plus DHD treatment pregnancies. (E) There was a decrease in fetal weight in response to stress that was further decreased in response to DHD supplementation. The results represent the mean ± SE for each sex group (*P < 0.05 as compared with control). (Student’s Independent T-test). Legend refers to Fig. 20A, B, D, E.
Stress challenge and stress plus DHD supplementation had an effect on fetal thymus on GD16.5.

The absolute number of thymic cells was increased in response to DHD-treatment, significantly in male offspring (Fig. 22A). The ratio of pre-selection (CD3⁺CD8⁺CD4⁺) to post-selection (CD3⁺CD8⁺CD4⁺) thymic cells was increased in prenatally stressed offspring, significantly in males, indicating a less mature phenotype. This was reversed with DHD supplementation, significantly in male offspring (Fig. 22B). There was a decrease in regulatory T cells within the CD3⁺ thymic cells population in response to stress challenge, which was significant in male and female offspring, and this was almost completely reversed by DHD supplementation, significantly in females (Fig. 22C).
Figure 22. Stress challenge and stress challenge plus DHD supplementation had an effect on fetal thymic development on GD16.5. Comparison of stress and control within sex groups. Fetal thymuses were harvested on GD16.5. Single cell suspensions were stained for CD3, CD4, CD8, CD44, CD25 and Foxp3. (A) Absolute number of thymic cells (1x10^5) was not significantly altered in response to stress challenge, but significantly increased in male offspring in response to stress challenge plus DHD (p<0.05). (B) Developmental ratio based on CD3 surface marker expression indicated that stress challenge significantly skewed the thymocyte population toward a less mature phenotype in male offspring (p<0.05), and this was reversed with progesterone supplementation, significantly in male offspring (p<0.05). Pre-selection double positive (CD25-CD44-CD3-CD8+CD4+) compared to post-selection double positive (CD25-CD44-CD3+CD8+CD4+) fetal thymic T cells. (C) Stress induced a significant decrease in the percentage of regulatory T cells (Treg) within the CD3+ thymocyte population which was significant in male (p<0.05) and female (p<0.01) offspring and was reversed with DHD supplementation, significantly in female offspring (p<0.05). Treg defined as CD3+CD4+CD25+++Foxp3+. The results represent the mean ± SE for each sex group (*P < 0.05, **P < 0.01 as compared with control) (Student’s Independent T-test).
Discussion

These experiments provide support for the proposed hypotheses, demonstrating that maternal stress during mid- to late gestation can have a programming effect on the development of the immune system by affecting fetal organ size and critical immune cell populations. HSC population size, migration and seeding are altered following maternal stress challenge. T cell development within the thymus is compromised, as indicated by a skewed CD3⁻:CD3⁺ ratio and lower Treg numbers. Further, these results provide support for the hypothesis that changes to the immune system can be ameliorated by progesterone supplementation in the form of DHD.

The information collected about basic pregnancy outcomes reflects previous studies utilizing this experimental model of sound stress challenge. Stress exposure at mid- to late gestation resulted in no change in either implantation or abortion rate between control, stress challenged and DHD supplemented animals. In contrast, studies that exposed dams to sound stress in early pregnancy had high levels of fetal loss despite having similar implantation rates. This supports the growing body of evidence in humans, mice and other species that there are multiple, specific windows of vulnerability during pregnancy that result in different pregnancy outcomes, including fetal programming. Other studies have also demonstrated that stress challenge leads to decrease in fetal weight (Personal Communication, M.E. Solano, January 2009). Surprisingly DHD supplementation did not prevent the decrease in fetal weight, in contrast to previous studies utilizing the same model (Personal Communication, M.E. Solano, January 2009). However, other studies have not seen any change in pregnancy outcomes in response to progesterone supplementation. Given that DHD supplemented animals had similar numbers of liver cells, HSC, thymocytes, Tregs and a similar CD3 ratio when compared to controls, it is possible that the stress-induced decrease in fetal weight is related to other factors. The influence of other hormones present at the feto-placental unit could cause a decrease in fetal weight that circumvents the progesterone pathway. For example, accumulating evidence indicates that altered levels of estrogen are linked to changes in fetal weight and immune cell development, among other outcomes.

The decrease in fetal organ size seen in the GD16.5 liver and thymus in response to maternal stress challenge is consistent with the central hypothesis, as it represents a compromise in fetal immune organ development. Successful fetal liver development is critical for fetal, neonatal and later life health, as it performs crucial functions in metabolic homeostasis, detoxification and endocrine regulation. Smaller liver size has been linked to functional consequences for disease risk, including cardiovascular disease in humans. Further, stress-induced adaptations may be related to impaired HSC function or impaired liver cell function, which could result in impaired HSC differentiation or impaired adult liver function. Studies have demonstrated that in utero exposure to toxic chemicals induces an altered HSC population, which could be linked to impaired thymic organogenesis and
T cell development. These chemically exposed HSC also had reduced reconstitution capacity when transferred into an irradiated host. In the fetal liver on GD16.5, the smaller organ size is accompanied by a larger percentage of HSC, but this results in the same absolute number of HSC in control and stress challenged offspring. This could be a compensatory mechanism by which this critical hematopoietic organ is maintaining equal numbers of seeding HSC at the expense of overall organ structure.

Thymic organogenesis is influenced by impaired HSC development and seeding to the thymus. Additionally, external programming factors influence thymic development. In human studies, infections or inflammation can lead to thymic involution, decreased thymic organ size and a severe reduction of thymocytes. Murine studies have also demonstrated that external factors, such as exposure to chemicals or toxins can result in fetal thymic atrophy, and alterations to normal patterns of thymocyte development. For example, exposure to low levels of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) or diethylstilbestrol (DES) can result in altered CD4 and CD8 expression, altering the balance of double negative (CD4⁺CD8⁻) to double positive (CD4⁺CD8⁺) T cells. This inhibited maturation is consistent with the altered CD3⁻:CD3⁺ maturation ratio observed in this project in response to stress challenge.

The fact that histological and morphological appearance of the fetal thymus was only slightly altered by prenatal stress challenge indicates that superficial structural development of the organ is not significantly compromised within the 48 hours post-stress challenge. Thymic epithelial cells (TEC) are crucial for correct T cell development, and specialized TEC subpopulations regulate specific stages of maturation and differentiation. Medullary TEC also have an important role in tolerance induction, regulated by a broad spectrum of genes that are under hormonal and epigenetic control, particularly Aire. Thus, it is possible that the cellular composition of the thymus is altered. The trend towards a decreased organ size combined with the clear changes within the thymocyte population indicate that the effects of stress challenge act directly on mechanisms that program the developing immune system. Whether this effect is mediated through interactions with TEC, epigenetics, altered HSC seeding or a combination of factors is unclear, and requires further analysis.

The consistent decrease in thymic cell numbers seen on GD16.5 is also consistent with the primary hypothesis. Having a small progenitor pool could limit the amount of variation that occurs during TCR rearrangement and selection, which would translate into a smaller number of mature peripheral thymocytes that have a more restricted antigen recognition repertoire. It could also be partially responsible for the altered Th1/Th2 balance seen in human and murine adult offspring with immune diseases in later life.

Furthermore, the decrease in thymocytes is accompanied by a delayed maturation status as indicated by a skewed CD3⁻:CD3⁺ selection ratio. As discussed by Godfrey et al, there are certain control points that are critical for correct T cell development. Expression of CD3 and the transition from the
pre-selection double positive to the post-selection double positive stage is one such point, and as previously mentioned it is vulnerable to external factors\(^{11,107}\). Additionally, it is coupled to TCR rearrangement, which is required for the development of a large T cell antigen-recognition repertoire\(^{118}\), and plays a critical role in mediating positive and negative T cell selection\(^{119}\). Further, a deficiency in TCR expression can lead to a block in thymocyte development\(^{120}\). Thus a decreased or altered antigen recognition repertoire could be explanatory for later immune system compromise seen in children and adults.

On GD18.5, the lack of differences between control and stress offspring suggests that as yet undetermined mechanisms within the prenatally stressed fetuses are compensating in order to attain equilibrium of immune development. The specific CD3⁻:CD3⁺ ratio was not statistically analyzed on GD18.5. Instead, the single positive CD3⁺CD4⁺CD8⁻ and CD3⁺CD4⁻CD8⁺ subsets were analyzed as an indication of T cell development and fetal immune competency. The significantly increased proportion of single positive CD8⁺ cells could indicate a delayed T cell emigration from the thymus. This could represent an aftereffect of the prolonged thymic seeding related to prenatal stress challenge. Examining the frequency and phenotype of recent thymic emigrants would provide insight into the association of prenatal stress challenge with a delayed emigration of mature, single positive CD8⁺ T cells from the fetal thymus.

If migration of mature, single positive CD8⁺ T cells to the periphery is occurring in control offspring but not in prenatally stressed offspring, this indicates a maturational delay and could account for postnatal immune compromise. Further, the relative numbers of each migrating T cell population could imply the distribution of T cell subsets and potential subsequent immune response bias in later life. Finally, analysis of thymic emigration patterns could reveal if seeding is occurring equally to all areas of the periphery.

Fetal development in mice and humans\(^{67,69,121-124}\) occurs in stages and disruption of this ordered progression has been shown to have negative repercussions for health in later life\(^{12,14,16,18,22-24,43,58,125,126}\). The nature of these postnatal consequences depends on the timing and duration of stress exposure\(^{11,13,101,102}\), and how well the fetus is able to recover from stress challenge.

The stress-induced changes seen on GD16.5 indicate that it is a potent programming factor being applied at a vulnerable time point. However, analysis at GD18.5 indicates that the developing fetus has attempted to adapt to this environmental challenge. Numerous studies have shown that it is this attempt to maintain developmental equilibrium that programs postnatal consequences, particularly in models of nutrient restriction\(^{22,23,127}\) and low birth weight\(^{48,49}\). Thus this effort to normalize the changes that occur during fetal programming events can be as critical as the challenges themselves. This has implications for neonatal healthcare, for example, in treatment of preterm or small for gestational age infants who will have different nutritional requirements as a result of their programmed predisposition towards conditions such as diabetes\(^{128}\), obesity\(^{129}\) or heart disease\(^{22,48}\). Understanding
the impacts of fetal programming will allow the provision of better health care in postnatal life.

Not all aspects of fetal development are returned to equilibrium, however, as demonstrated by the persistent decrease in regulatory T cells in response to stress challenge. While the distribution of T cell populations may normalize through a compensatory mechanism, the loss of HSC seen at the transition from fetal liver to bone marrow on GD18.5 indicates that there is an adverse effect occurring within the fetus. In an attempt to compensate for the delayed T cell maturation, it is possible that a higher than normal number of HSC are required to seed into the thymus as CD4 or CD8 progenitors, thus decreasing the number available in the bone marrow for other requirements and potentially not making the contribution to the regulatory T cell population.

Further evidence for delayed T cell maturation is represented in the reverse CD3^{−}:CD3^{+} ratio seen on GD18.5. Given that there are two waves of HSC seeding to the thymus, it is possible that this captures the beginning of the second wave in control offspring and the delayed end of the first wave in stress challenged offspring. Another possibility is that cells from stress challenged offspring are overcompensating for the delay in maturation seen in GD16.5, as mentioned above, and responding with an overshooting maturation that could result in CD3^{+} T cells that have impaired function.

Sex-specific differences in offspring response to stress challenge never reached significance, but displayed an interesting trend on GD18.5. Fetal organs were slightly smaller in female offspring, and this was accompanied by differences in individual cell populations, specifically the ISP, pre-DP and CD4+ SP populations. Given that sex-specific effects are seen pre- and postnatally in human and murine studies (Personal communication, C. Bruenahl, December 2010) this could be an early indicator of these changes. Furthermore, it is well established that fetal development has sex-specific components that are influenced by hormones. Given that there are stress-induced alterations in maternal endocrine levels it is possible that these changes have differential effects on the fetus depending on its sex.

**Limitations**

A major limitation during these experiments was the low cell number seen in fetal organs. This cell number is confirmed in previous studies, however the majority of other studies pooled their tissue when analyzing fetal samples. Given the strong postnatal evidence of a sex difference in response to prenatal stress (Personal Communication, C. Bruenahl, December 2010) and the physiological differences in development between male and female offspring, tissue from individual fetuses was analyzed independently. While this allowed for a detailed examination of the sex-specific effects of prenatal stress challenge, it limited the possibilities for FACS staining. In the thymus, low cell numbers restricted phenotypic analysis and so other markers of interest (i.e. MHC II, TCRβ, TCRγδ) could not be included. Looking at MHC II or TCR expression would allow further investigation of the effect of stress on the critical “control points” in T cell
An attempt was also made to analyze HSC populations in the fetal spleen, but the cell numbers present after tissue isolation were prohibitively low. The spleen is a hematopoietic organ in late murine gestation, and remains an important lymphoid organ throughout adult life. It has a critical role in antibody production, mediating interactions of DCs, T cells and B cells, facilitating somatic hypermutation of B cells, monitoring for systemic antigens and pathogens, and functioning in immune homeostasis. In particular, fetal spleen stroma has a vital role in the differentiation and maturation of splenic macrophages. It has a critical role in innate and adaptive immune responses, and so a compromise during fetal development could result in immune compromise in later life.

Similarly, the fetal bone marrow was not examined on GD16.5 due to technical limitations. The small organ size and amount of extraneous tissue hindered the processing and made it complicated to properly purify the samples for true single cell isolation. This caused problems with FACS staining, which became contaminated with non-specifically stained debris, and with acquiring samples in the FACS machine. While the fetal bone marrow does not become critically important in fetal immune cell development until GD18.5, it would have provided a helpful baseline from which to analyze kinetic data for later timepoints.

Other aspects that were not included in these experiments included weighing fetal organs as another assessment of organogenesis and development. As previously indicated, fetal immune organ size and weight can be critical indicators of fetal health and later life fitness. For example, the self-renewal and pluripotent capacity of HSC are dependent upon interactions with the stromal microenvironment.

A further limitation was reflected in the groups selected for the progesterone experiment series. There was no sham-injected group, which would have provided a control for the effect of any potential stress related to the injection that would have altered the results from the stress plus DHD supplementation group. This control has been present in previous studies from our group, and has demonstrated that there is no significant effect of injection on fetal outcomes relating to stress challenge.

Another limitation was the low population number for the GD16.5 experiments. It is possible that increasing the number of fetuses per gender per group would reveal the presence of any type II errors, which could be masked by a small population size. For example, there is a significant decrease in the number of fetal liver cells in response to stress challenge in female but not male offspring, despite similarly distributed populations.

Additionally, there is a minor discrepancy between the liver cell number and HSC population on GD16.5 between the original experiment (Figure 9) and the DHD experiment (Figure 21) that reproduced those results. It is possible that the change in animal facility could have contributed to the minor difference seen in these populations, despite all attempts to reproduce the same conditions. This limitation is relevant due to the potential importance of...
HSC seeding from the liver to the thymus in order to provide T cell progenitors, and would need to be addressed in further experiments.

**Implications and Future Directions**

This research provides a platform for further investigations to fully characterize the effects of stress on the fetal immune system and how these changes persist into postnatal life.

To date, few studies have assessed the longitudinal kinetics of fetal immune development in the context of an environmental challenge. While the changes occurring during fetal development are well characterized, little information exists about the influence of external factors such as stress challenge. Thus making a connection between fetal programming factors and adverse adult outcomes has not been concretely established.

Of particular interest in establishing the link between changes seen in fetal immune ontogeny and adverse postnatal outcomes is the persistent decrease seen in Tregs on GDs 16.5 and 18.5. Analysis of adult outcomes of the same stress challenge model revealed that not only are these offspring more prone to develop airway hypersensitivity, they display a decrease in Treg frequency (Personal Communication, C. Bruenahl, December 2010). Human studies have indicated that neonatal and postnatal Treg function can have a dramatic impact on immune regulation and development of chronic immune diseases in later life. Given that T cells are long lived, the decrease in Tregs seen in fetal life could account for the permanent impairment seen in these adult offspring. Furthermore, studies in humans have also implicated a major role for fetal Tregs in feto-maternal interactions that lead to programming, and have demonstrated that a portion of fetal Tregs persist as memory T cells into adulthood. In a larger perspective this has critical implications in the study of fetal and maternal microchimerism, which could be one of the primary mechanisms that contributes to fetal programming.

Bi-directional trafficking of fetal and maternal cells across the placenta is well-established in humans and mice. Studies have shown that cell trafficking at the feto-maternal interface is tightly regulated, most likely to mediate immunotolerance. It is known that fetal cells are able to interact with and modulate the maternal immune system, and current research is now focused on the role of maternal cells in the fetus. Mold et al were able to show that maternal alloantigens are responsible for inducing human fetal Treg cells in utero, which can induce antigen-specific tolerance and most likely function in regulating postnatal immune responses. Another group has reported the appearance of maternal cells in murine fetal bone marrow. They suggest that not only do these cells have a role in promoting hematopoiesis, but that proper fetal immune development is critical for correct maternal cell distribution. When considered together, these findings have tremendous implications for the role of maternal influences on the developing immune system. It is possible that maternal cells present in the fetus are one of the mechanisms for the consequences of prenatal stress. A compromise at any
stage of microchimerism – maternal cell development, placental trafficking, maternal-fetal cell interaction – could result in impaired immune function and the development of chronic immune disease. Further investigation into the effects of stress on microchimerism is required to understand how these interactions work and to develop strategies to protect them.

A logical next step in this research would be to examine Treg functionality using proliferation and suppression assays. This would provide information about whether the Treg population is simply decreased in number or ability to proliferate, which then limits their function, or whether their immunoregulatory functions have been impaired or inhibited in response to prenatal stress challenge. This is of major clinical relevance, given that Tregs have a critical role in regulating many, if not all, immune diseases.

Testing functionality of HSC or other T cell populations would also be of interest. While this data demonstrates that immune cell population frequencies are decreased, this does not indicate whether their ability to mediate correct effector functions is impaired. It is equally as important to have a sufficient number of immune cells as it is to have cells that function properly. For example, a smaller HSC population that can still correctly differentiate into lymphoid, myeloid or tissue-specific progenitors represents a less dramatic consequence of fetal programming than an HSC population that cannot differentiate. Further, sequencing TCR junctional regions to explore the extent of T cell diversity would provide an opportunity to evaluate whether prenatal stress challenge reduces T cell diversity and thus mediates negative health outcomes in later life.

Functionality could be tested using a combination of different techniques, including the proliferation and suppression assays mentioned above. For example, reconstitution of irradiated host bone marrow using HSC from stress-challenged or control offspring would also indicate whether HSC are functioning properly. Alternatively, transferring stress-challenged or control lymphocytes into T cell deficient mice (i.e. RAG2-/-, SCID, Nude) and then exposing them to a variety of pathogens could reveal how well these cells are able to respond. Testing the functionality of stress-challenged immune cells would provide valuable insights into the mechanisms behind chronic immune diseases.

Examining other immune parameters that could have relevance during gestation would further clarify and expand the limited knowledge available about the effects of stress on the fetal immune system. Analysis of apoptosis in the fetal thymus could provided insight into thymic development, given that studies have shown increased apoptosis leads to thymic atrophy and T cell impairment.

Additionally, given that the immune system does not function as populations of different cells operating in isolation, looking at other subsets of immune cells will provide a more comprehensive understanding of the impact of prenatal stress challenge. Early pilot data indicated the presence of CD8+CD11b+ cells in the fetal bone marrow (data not shown), and it is well established that B cell and NK cell development can occur during the fetal period. It is highly possible that prenatal stress challenge would alter or impair
the development of these and other innate immune cell populations, as well as other fetal immune organs.

Analysis of other time points will provide a more detailed overview of the kinetics not only in the T cell and HSC populations on specific gestational days but also of the overall patterns of development and impairment seen in response to stress challenge. For example, having more time points will provide a better understanding of the role of the two waves of thymic seeding by HSC and the consequences if this process is disrupted. If possible, it would be ideal to analyze fetal tissue twice each day of mid- to late gestation to clearly identify when stress challenge begins to influence immune system development, and what specific control points are affected.

Another critical period to investigate is the immediate postnatal period, which is characterized by mucosal colonization and further immune development. This is also a time point frequently studied in human investigations, as it is less complicated to acquire cord blood than human fetal tissue, and so will provide a basis for comparison and clinical relevance.

Another interesting avenue for continued research would be to further investigate the role of HSC in terms of colonization of other areas of the fetus and neonate, such as the blood, spleen and postnatal bone marrow. A study that analyzed the long-term impact of an altered fetal HSC population could capture the relevance of prenatal stress on postnatal HSC function, such as gut mucosa colonization, size and functionality of the HSC population in the bone marrow, and whether their self-renewal and reconstitution potential is compromised.

Epigenetics have a critical role in the development, differentiation and maturation of immune cells. This has been demonstrated for HSC as well as T cells, and is evident on a larger scale in the context of overall health or disease. Of particular interest is the methylation status of the Foxp3 locus, which is methylated in all T cell subsets except naturally occurring Tregs at the highly conserved CpG-rich island. Thus Foxp3 expression and the Treg phenotype are under epigenetic control. Given that stress can induce epigenetic changes in affected offspring (Personal Communication, C. Bruenahl, December 2010), it is possible that the decrease seen in fetal Tregs could be a result of epigenetic changes or could be compounded by altered methylation status in later life, adding to health complications.

Maternal endocrine factors also impact the fetus. It is well established in humans and mice that stress can induce a decrease in maternal progesterone levels, and that this has an effect on the fetus. Interestingly, a recent study using this experimental mouse model has demonstrated that increased offspring airway responsiveness can be abrogated by progesterone supplementation (Personal Communication, C. Bruenahl, December 2010). This suggests that progesterone has a protective effect against impaired offspring immune responses, including immune system development. Progesterone has an important role in pregnancy establishment and maintenance. These functions are mediated by a variety of different factors, primarily working through the progesterone receptors, and through interactions with immune cells and uterine tissue. The progesterone
receptors control a variety of different genes, such as galectin-1\(^{59}\), each with specialized functions in promoting feto-maternal tolerance and a stable uterine environment\(^{152}\). Also, binding of progesterone receptors expressed on maternal cells induces progesterone-induced blocking factor (PIBF), a mediator of progesterone that blocks non-uterine natural killer (uNK) activity and induces Th2 cytokine production\(^{153}\). Given that progesterone has a critical role in pregnancy success, the endocrine effects that create a suitable in utero environment could impact fetal development in a variety of ways. Whether by altering maternal immune-endocrine homeostasis\(^{20,96,150}\) or impacting fetal outcomes\(^{18,64,97}\), it is likely that low progesterone levels influence fetal programming.

The critical influence of progesterone on pregnancy maintenance suggests that other maternal factors may also have a crucial role. It has been demonstrated that maternal estrogen levels are increased in response to stress challenge (Personal Communication, M.E. Solano, January 2009). Interestingly, it has also been shown that increased estrogen levels impair thymic size and cellularity, and inhibit T cell maturation in the thymus, retaining cells at the DN1 stage\(^{105}\). Considered together, these data suggest that increased levels of maternal estrogen may have an equally important role in mediating the impaired T cell development seen in these experiments.

There is accumulating evidence that another maternal factor, fibromyalgia syndrome-like tyrosine kinase 3-ligand (Flt3-L), can cross the placental barrier\(^{154}\) and bind to Flt3 receptors present on fetal HSC\(^{155}\) and TEC\(^{156}\). This receptor-ligand interaction is critical for HSC differentiation\(^{155}\) and maintenance of early stage of T cell development\(^{156}\). Flt3-L has also been shown to act as a self-renewal signal for early thymic progenitor cells\(^{157}\). As such, it is possible that maternal levels of Flt3-L could be altered by stress challenge which could then influence maturation of fetal and, subsequently, neonatal immune cells like T cells or DCs\(^{43}\).

Another critical feto-maternal interaction occurs at the placental interface. Maternal glucocorticoids are upregulated during stress, and can cross the placenta barrier due to their lipophilicity\(^{158}\). The fetus is only protected from these effects by the enzyme 11-beta-hydroxysteroid dehydrogenase type 2 (11\(\beta\)-HSD2), which converts maternal glucocorticoids (cortisol in humans, or corticosterone in mice) to the inactive form (cortisone or 11-dehydrocorticosterone)\(^{158,159}\). Either an upregulation in maternal glucocorticoids that overwhelms this barrier or a decrease in 11\(\beta\)-HSD2 levels can result in excess glucocorticoid exposure\(^{160}\). Experimental evidence in mice\(^{158,160}\), humans\(^{159,161}\) and rats\(^{162}\) has demonstrated that this has consequences in later life, such as hypertension, metabolic disease, and behavioural problems\(^{161,162}\).

Thus synthetic progesterone supplementation during pregnancy could be used as one of many possible therapies to treat or reverse increasing incidence of chronic immune disease without needing postnatal interventions. While there are many other steps required before recommending clinical prescriptions, these data contribute to a growing body of basic science evidence for the numerous benefits of progesterone supplementation.
Strategies are urgently needed to promote maternal and fetal health without restricting maternal activities during pregnancy.

Conclusion

These results, when considered in the context of currently available literature, support the role of prenatal stress challenge as a fetal programming factor (Fig. 19). The impaired immune development seen in response to stress challenge indicates a potential cause for the increasing incidence of chronic immune diseases seen in the last five decades. Thus it reaffirms that maternal stress during pregnancy should be considered a primary risk factor for later life diseases. Furthermore, this research supports the role of progesterone as a supplement during pregnancy as part of a larger, more comprehensive prenatal maternal health regimen. Thus this research is highly relevant in both basic science and clinical contexts, and provides a strong platform for further investigations that could potentially lead to novel maternal and fetal health strategies in the prenatal, perinatal and postnatal periods.
Figure 23. Summary of the effects of stress on the development of the fetal immune system.
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Appendix

Figure A1. (A) Overall distribution of T cell populations in the fetal thymus on GD16.5 and GD18.5. Sections represent proportion of each population within total thymocytes. Population maturity increases in a clockwise direction, beginning with DN1 and ending with SP CD8\(^+\).

Figure A1. (B) Comparison of the overall distribution of T cell populations in the fetal thymus on GD16.5. Sections represent proportion of each population within total thymocytes. Population maturity increases in a clockwise direction, beginning with DN1 and ending with SP CD8\(^+\). Stress challenge resulted in significant differences in the DN4 and ISP populations.
Figure A1. (C) Comparison of the overall distribution of T cell populations in the fetal thymus on GD18.5. Sections represent proportion of each population within total thymocytes. Population maturity increases in a clockwise direction, beginning with DN1 and ending with SP CD8+. Stress challenge resulted in significant differences in the DN1, post-DP and CD8 SP populations. The results represent the mean ± SE for each sex group (*P < 0.05 as compared with control). (Mann-Whitney U test).