BIRTH DEFECT AMELIORATION AND PLACENTAL CYTOKINE EXPRESSION IN MNU-EXPOSED DAMS TREATED WITH IFN-γ

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(ABSTRACT)

Each year, 7.9 million babies are born with birth defects. Seventy percent of those could be prevented, ameliorated, or repaired; yet 3.2 million children still die by the age of three (March of Dimes Global Report 2006). We have found that non-specific maternal immune stimulation with the cytokine interferon-gamma (IFN-γ) can successfully ameliorate some of these defects in the C57BL/6N mouse model. We have observed a reduction in the distal limb malformations syndactyly, polydactyly, and webbing by 47%, 100%, and 63% respectively when IFN-γ is given 2 days prior to MNU administration. We have also observed that IFN-γ works at the placental level to protect against MNU-induced damage. Trophoblast loss and associated cytokine alterations occur in gestation day (GD) 14 placenta following GD9 MNU exposure, showing that fetal-maternal communication can be hindered due to MNU. In the labyrinthine layer of the placenta, we observed multifocal fibrinous necrosis of endothelial cells due to MNU, however IFN-γ almost completely protected the trophoblast and endothelial cells when given to the dam as an immune stimulant. To determine the genes participating in these processes, gene microarray studies were conducted. Hepatocyte growth factor (HGF), interleukin 1 beta (IL1β), and insulin-like growth factor 2 (IGF2) were elucidated as genes that were significantly expressed in GD12 placenta. These genes are similar in that they are all connected to the Jak-Stat signaling pathway. These findings provide a

possible mechanism for birth defect reduction by maternal immune stimulation with IFN-γ in MNU-challenged mice.

Dedication: To my husband and best friend, Benjamin Scott Laudermilch.

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Steven D. Holladay provided research oversight and guidance contributing to the data within all of the following chapters. He administered expertise and advice in developmental immunotoxicology, statistics, and anatomy. He helped plan and troubleshoot problems with the experimental procedures, and provided literary criticism.

William R. Huckle imparted research oversight and guidance contributing to the data within chapters I, II, IV, and V. He administered expertise and advice in molecular biology and statistics. He helped plan and troubleshoot problems with the experimental procedures, and provided literary criticism.

Jolynne R. Tschetter offered research oversight and guidance contributing to the data within chapters I, II, IV, and V. Her expertise and advice in molecular biology helped in the planning, experimentation, and interpretation of the studies. She was also an asset to the work by providing literary criticism.

Stephen R. Werre contributed statistical consultation and development for the data represented in chapters IV and V. His expertise in biostatistics with relation to microarrays helped direct our pathway to interpretation of the data.

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Figures:

Tables:

Abbreviations/Symbols: A listing of abbreviations or symbols used in the body of the document

IFN- γ:	Interferon-gamma
MNU:	Methylnitrosourea
Th1:	T-helper 1
Th2:	T-helper 2
HGF:	Hepatocyte growth factor
IL1β:	Interleukin 1 Beta
IGF2:	Insulin-like growth factor 2
Ifrd1:	Interferon-related developmental regulator 1
Fgf3:	Fibroblast growth factor 3
Jak:	Janus activating kinase
Stat:	Signal transducer and activator of transcription
GD:	Gestation day
NK:	Natural killer cells
uNK:	Uterine natural killer cells
MLAp:	Mesometrial lymphoid aggregate of pregnancy
DB:	Decidua basalis
PE:	Preeclampsia
IUGR:	Intrauterine growth retardation
SGA:	Small for gestational age
gKO:	IFN-γ knock out
NOC:	N-nitroso compounds
ROS:	Reactive oxygen species
SPF:	Specific pathogen free
NO:	Nitric oxide
RT PCR:	Reverse transcriptase polymerase chain reaction
SOCS:	Supressor of cytokine signaling

CHAPTER I

INTRODUCTION:

Each year, 7.9 million babies are born with birth defects. Seventy percent of those could be prevented, ameliorated, or repaired; yet 3.2 million children still die by the age of three (March of Dimes Global Report 2006). The most common types of musculoskeletal defects are lower and upper limb reductions, where bones and cartilage are underdeveloped or completely missing. In the United States, there are 2.2 babies born with lower-limb reductions per 10,000 live births and 4.4 babies born with upper-limb reductions per 10,0000 live births (March of Dimes, National Perinatal Statistics). The studies herein examined birth defects of musculoskeletal classification and included hypoplasia of the long bones such as in phocomelia and achondroplasia; digital malformations such as syndactyly, polydactyly, oligodactyly, webbing, and clubbing; and overall reduced body size.

Many factors, including genetics, lifestyle, maternal age, and prenatal care and nutrition contribute to the occurrence of birth defects. Various vitamins, minerals, immune modulators, and exercise and diet regimens have been employed to help decrease fetal defects and preterm birth. A key site in the maintenance of a viable pregnancy is the feto-maternal interface (uterus, decidua, spiral arteries, placenta). Proper placental development as well as adequate uterine architecture insures that the exchange of nutrients, gas, and immunological signals is achieved between mother and conceptus. Immune stimulants have been shown to reduce fetal birth defects and

placental maldevelopment. The mechanism of the amelioration of these effects due to immune stimulation is unknown.

IFN-γ is a non-specific immune stimulant that can be harmful or helpful to pregnancy, depending on concentration and time of administration. In our studies, IFN-γ has demonstrated the ability to ameliorate teratogenic effects of methylnitrosourea (MNU), a potent mutagen, carcinogen, and teratogen. IFN-γ was able to reduce fetal malformations as well as well as work at the placental level to protect against MNU-induced histopathological damage. These reduced birth defects and improved placental architecture as a result of IFN-γ administration have led us to believe that immune modulation promotes proper placental development and thereby reduces fetal maldevelopment. To determine the main genes and pathways regulating the immune-mediated birth defect amelioration, we also investigated placental gene expression.

CHAPTER II

LITERATURE REVIEW:

The Feto-Maternal Interface

The murine period of embryogenesis is an excellent model for teratogenic studies due to the similarity of placental architecture with humans. The murine placenta is discoid in shape and is classified as an invasive, interstitial, or decidual membrane. From maternal side to fetal side, the interface begins with the external or serous portion of the maternal uterus called the visceral peritoneum. Next, is the muscular portion of the uterus called the myometrium, providing parturition capabilities. The most internal region of the uterus is the uterine mucosa or endometrium, consisting of connective tissue, glands, and vessels, and covered by a layer of epithelial cells. This epithelium faces the embryonic epithelium of the fetal trophoderm or visceral yolk sac placenta. These two layers of epithelium are often referred to as being contiguous. The visceral yolk sac tightly covers the discoid placenta that consists of the mesometrium, trophospongium, labyrinth, and then chorionic plate. The amnion is the next layer, and is surrounding the fetus.

The murine placenta is hemotrichorial, meaning that there are three different trophoblast layers separating the maternal and fetal blood. Within the labyrinthine placenta are fetal capillaries that are surrounded by trophoblasts. There are three types of trophoblasts: giant cells or spongiotrophoblasts, which penetrate maternal tissue and grow into maternal blood vessels and mix with maternal decidual cells; and syncytiotrophoblasts and cytotrophoblasts which make up the walls of the labyrinthine

tubules. These trophoblasts are bathed in maternal sinusoidal blood. Maternal vessels penetrate the central region of the mesometrial side of the placenta; the uterine artery will then branch to form spiral arteries. The umbilical cord leaves the chorionic (fetal) side of the placenta and makes connection with the fetus.

The Role of Placental Protection

A healthy placenta generally results in a healthy baby. The placenta serves several functions to promote homeostasis in the developmental environment of the pup. Besides the transport of oxygen and soluble nutrients to the embryo, and wastes away from, the lipid membrane of the placenta provides a protective barrier to antigenic attack by allowing passive immunity to cross from mother to conceptus. Studies have shown that immunoglobulins, viruses, and dyes can transit the epithelial cells of the endometrium and visceral yolk sac [1, 2]. The placenta can also provide a buffer from some unhealthy attacks of/on the dam.

Pathological assessment of the viability of the cells that conduit oxygen and nutrients to the fetus and the amount of blood flow within the placenta can often reveal the nature of the environment of the developing pup. Examination of the myometrium, trophoblast giant cells and syncitiotrophoblasts leads to conclusions involving the production of growth factors and regulation of placental development. Glycogen-rich trophoblasts indicate the metabolism and amount of energy storage available to the fetus, and the vascularization of the labyrinth layer can indicate the amount and condition of maternal-fetal circulation exchange [3].

Aschkenazi et al. found that the direct administration of IFN-gamma on trophoblast cells resulted in fewer viable trophoblasts. However, Laudermilch et al. found that indirect administration of IFN-gamma by way of the maternal immune system protected trophoblasts from MNU-induced trophoblast loss [4].

Important Disease Conditions of the Placenta

Two important disease conditions that occur at the feto-maternal interface are pre-eclampsia (PE) and intrauterine growth retardation (IUGR). Pre-eclampsia is a condition that occurs in late gestation and presents the signs and symptoms of edema, elevated blood pressure, proteinuria, headaches, and weight gain. PE partially inhibits nutrient and gas exchange between the mother and fetus and can result in fetal death. Maternally, platelet aggregation and vasospasm can lead to thrombus formation, renal necrosis, and impaired glomerular filtration. PE often results in pre-term delivery of the fetus due to the placental instability.

IUGR is a condition in which the placental membranes do not adequately develop in size and thus restrict the nutrient supply to the embryo and impair development. This can be the result of many things, including infection, placenta previa (low lying placenta), placenta abrupta (premature placental detachment from uterus), chronic maternal disease or genetics. IUGR is characterized by smaller-than-normal fetuses termed “small for gestational age” or SGA. These babies also have decreased fetal weight and present as thin, with pale and dry skin and a thin and dull umbilical cord.

Cytokines in Pregnancy

Cytokines are small peptides or glycosylated proteins produced mainly by CD4+ T cells that are locally and transiently secreted by various cells in order to regulate the immune and endocrine systems. The cytokines present at the maternal-fetal interface are mainly located in the placenta and exert a local action by autocrine and paracrine loops [5]. A study by Daher et al. suggested that gene polymorphisms in gestational cytokines might contribute to the pathogenesis of recurrent pregnancy loss [6].

Trophoblasts and villous core stromal cells are the proposed primary sources of the placental cytokines [7] and trophoblasts are believed to control the polarization of maternal immune effectors and cytokine profiles at the fetal-maternal interface [8]. T-helper cytokines regulate trophoblast differentiation. Cytokine levels are highest during the first week of pregnancy with diminishing levels to the point of immunodepression thereafter [9].

The most common cytokines in pregnancy are interleukins-1, 4, 5, 6, 8, 10, 13, and 18 (IL-1, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-18), transforming growth factor-β (TGF-β), platelet derived growth factor (PlDGF), monocyte chemotactic peptide-1 (MCP-1), RANTES (Regulated on Activation and Normally T-cell expressed and presumably Secreted), and Leukemia inhibitory factor (LIF). IL-4 is the most common cytokine in the placenta and induces the Th2 response along with IL-10. The Th2 cytokine profile promotes trophoblast proliferation and differentiation and high levels of IL-10 protect the placenta from ischemic damage. The Th1 cytokine profile involves IL-2, IFN-γ, and TNF-α [10]. These cytokines reduce trophoblast differentiation and

invasion of spiral arteries as well as induce apoptosis in the trophoblast cells, which are trademark pathologies of pre-eclampsia [10]. VEGF is the most common angiogenic growth factor that is expressed by trophoblast giant cells. TGF-β also induces the Th2 profile. CSF-1 is able to traverse the placenta and is important in embryonic development. IFN-γ and G-CSF also are able to cross the placenta. Via interactive communication signaling of the cytokines, the maternal-fetal interface is maintained at a homeostatic level.

The Immunology of Pregnancy

The development of an embryo inside the mother is regarded as a foreign allograft to the maternal immune system. Antigenic stimuli cause CD4+ T lymphocytes to produce two different forms of T helper cells, Th1 and Th2. Th1 cells mediate the cell-mediated proinflammatory response and Th2 cells elicit the antibody-mediated humoral response while down-regulating the cell-mediated response. T-cells then produce or activate antigen-attacking cells that are recognized by major histocompatability complex (MHC) antigens on their surface. In humans, it was found that trophoblasts do not express the human leukocyte antigen (HLA) class 1 antigens and therefore do not undergo attack from maternal T cell-mediated alloreactivity [11]. However, the body's next defense mechanism would come from natural killer (NK) cells, which lyse cells lacking HLA1 molecules on their surface. The discovery of the HLA-G molecule localized to anchoring extravillous trophoblasts helped to explain the permissible presence and invasion of the trophoblasts in spiral arteries and interstitium [11-13].

Abrahams et al. reported that trophoblasts promote local immune suppression and thus evade the maternal immune system [14]. Pregnancy is also permitted because the Th2 response is regulated by various maternal cytokines that maintain a balance between the anti-inflammatory Th2 cytokines and the pro-inflammatory cytokines of the Th1 response. Without this balance, the maternal immune system would activate maternal leukocytes and macrophages to attack the fetus and placenta. The Th2-type immunity is essential for embryonic implantation, placentation, placental development and fetal growth [15]. Hayakawa et al. found that a dominance of Th1 or Th2 immunity could cause murine fetal absorption and an experimental form of pre-eclampsia [16].

The dramatic increase in progesterone levels that accompanies ovulation and the hormonal maintenance of pregnancy contributes to this immunological paradox. Pharmacological doses of these glucocorticoids determine the type of interleukin produced by T cells, according to Daynes and Araneo, inhibiting IL-2 and promoting IL-4 production [17]. Saito found that Th1 cytokine levels decreased as Th2 levels increased as the uterus was prepared from implantation [18]. It has also been reported that progesterone causes Th0 cells to change into Th2 cells [19].

The Effects of MNU

N-Methylnitrosourea (MNU, $CH_3NNOCONH_2$) is an N-nitroso compound (NOC) that acts as an alkylating agent. It is commonly used in research to induce carcinogenesis or teratogenesis in laboratory animals; the agent is also genotoxic. Alkylating agents such as MNU are ubiquitous, although often in low levels. NOCs are commonly

encountered in nitrate-cured meats, seafood products, malt beverages, cosmetics, and rubber products [20, 21]. Endogenously formed NOCs may contribute to the greatest exposure, by transformation of amides at a low pH in the stomach. However, no studies have shown a correlation between the incidence of human cancer and endogenous NOC exposure. At low levels, the body is normally able to enzymatically metabolize the alkylating compounds, however, at higher levels, and especially during gestation, these agents can exert their teratogenic effects.

The mechanism of action of alkylating agents in the body involves the methylation of nitrogen and oxygen in DNA bases which may result in strand breaks or interstrand cross links [22]. Bochert et al. proposed that DNA alkylation in embryonic tissues was the primary cause of birth defects [23]. The teratogenic effects of DNA damage involve interruption of the cell cycle, thus confounding apoptosis, and increasing the production of reactive oxygen species (ROS). A more specific mechanism of action was presented by Jacoby, showing that MNU induces G-to-A transitions at codons 12 and 13 of the K-ras gene in rat colon tumors [24]. The Ras proteins are from the G-protein family and possess GTP/GDP binding capacity. They are important mediators of growth-factor induced responses by acting as a molecular on/off switch. Ras is located upstream of mitogen-activated protein kinase (MAPK) whose activation ultimately upregulates the expression of cyclin D and initiates the mitotic cycle [25]. A point mutation in codon 12 (mutation of the ras gene) will dramatically lower the GRPase activity of the protein and thus cause Ras to be in the permanently active GTP-bound state, which will lead to uncontrolled proliferation and transformation.

Once DNA damage occurs, repair mechanisms encoded on the gene O6-alkylguanine-DNA alkyltransferase (AGT or MGMT) carry out base excision repair, direct base repair, and nucleotide excision repair [22]. These mechanisms limit the lethal effects of alkylating substances in normal mice and humans but do not correct all of the possible detriments [26]. Subsequently, oncogenesis [27], teratogenesis, and premature aging can occur. A study by Hsieh and Hsieh found that DNA adducts do find their way into the feto-maternal interface in an aflatoxin B1 study examining human placenta and cord blood [28]. In another study continuing the investigation of the embryotoxic affects of MNU, Tetsuji Nagao and colleagues experimentally proved that MNU directly affects the developing pre-implantation embryo by causing gross malformations in the structural development [29]. It is possible that either the fetal gene hardware for DNA-lesion-repair is neither fully active nor sufficient to protect embryos from the teratogenic effects of alkylating compounds, or excess reactive oxygen species are produced at certain points of gestation that may predispose the fetuses to developmental deformities. It has been found that in early gestation (day 1) DNA breakage is maximal in the immediate hour following MNU administration and rapidly declines thereafter. However, day 11 administration of MNU causes many more DNA strand breakages [30]. Therefore, it is speculated that midgestational exposure to MNU (e.g. GD9 in this study) also leads to more DNA strand breakages and subsequent manifestation of MNU's teratogenic effects.

Immunologically, a study by Tarr and colleagues demonstrated that the cell-mediated-immune system and hematopoietic system were markedly suppressed for 3 months and 3 weeks, respectively, after a non-carcinogenic dose of MNU was given to

adult specific-pathogen-free (SPF) cats [31]. The authors were interested in the effect of MNU in cats because of their increased sensitivity (mortality) to MNU at rodent tumorigenic doses, and lower doses have been found to increase susceptibility to Feline leukemia infection and disease in SPF cats. The results from Tarr et al. showed prolonged cutaneous allograft retention time, decreased lymphocyte blast transformation response to mitogens and antigen, and a reduced number of absolute erythrocyte-rosetting T-cells in the peripheral blood. In the hematopoietic system, lymphocytes and neutrophils decreased peripherally, bone marrow cell counts were abnormally low, and arrested growth of neutrophilic and erythroid precursors was observed [31].

Grossly, the repercussions of fetal teratogen exposure appear as various defects depending on the time of application during gestation. Iannaccone et al. showed that MNU exposure to blastocysts resulted in an increased fetal resorption rate [32]. Exencephaly, cleft palate, and malformed vertebrae can be observed in fetuses treated with MNU on GD0-3 because these organs are undergoing rapid development at the time of teratogen exposure [29, 33]. Digital deformities are seen in dams treated with MNU GD9-12 [34, 35]. Also following MNU treatment on GD12, reduced fetal weight and brain mass can be observed in rat fetuses [36]. GD13 MNU treatment results in microcephaly [37] and retinal degeneration can be seen in mice treated with MNU on GD11-17 [38, 39].

Immune Stimulation with IFN-γ

Maternal immune stimulation has been shown to ameliorate various teratogenic effects. Recent studies have employed several nonspecific immune up-regulators such as Freund's complete adjuvant (FCA), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFN-γ), and butylated hydroxytoluene (BHT) [35, 40, 41]. Via various unknown mechanisms, these immune stimulants have demonstrated protection against the many developmental defects previously noted. The immune interaction at the maternal-fetal interface has been of interest due to recent research showing that immune stimulation can protect against damage to the placenta during toxic induction of birth defects [35, 41]. IFN-γ is a non-specific immune stimulant that can be harmful or helpful to pregnancy, depending on concentration and time of administration. Chaouat et al. found that it is abortifacient at high doses in synergy with TNFα [42]. However, Ashkar & Croy identified a crucial spike of IFN-γ at implantation and determined that it is necessary for local vascularization at the fetomaternal interface [43-46]. Work by Munn & Mellor found that IFN-γ induces antigen presenting cell expression of indoleamine 2,3 deoxygenase, which catabolizes tryptophan and results in decreased T-cell proliferation [47-49].

IFN- γ receptors are abundant on the surface of placental trophoblasts. It is proposed that in PE, placental trophoblasts fail to establish an early-to-late switch with respect to IFN-γ and IFN-γR2 (receptor) expression [8]. Spontaneous recurrent abortion has been linked to vascular endothelial attack and trophoblast degeneration due to the synergistic effects of IFN-γ and TNF-α [8]. IFN- γ can transduce signals from one cell type to the other through diverse effects; it also causes a wide range of cellular responses

including proliferation, apoptosis, leukocyte endothelial interactions, upregulation of nitric oxide (NO) production in macrophages (thereby increasing ROS), tryptophan metabolism, and mediation of MHC class I and II complexes [8, 50].

IFN-γ KO mice

IFN-γ KO mice (gKO) were developed in 1993 and provided evidence for the importance of IFN-γ in the function of several murine immune cells. Although they develop normally, IFN-γ-deficient mice exhibit impaired production of macrophage antimicrobial products as well as reduced expression of macrophage MHC class II antigens [51]. gKO mice are healthy in pathogen-free environments, but have an increased vulnerability to intracellular infections due to the lack of IFN-γ-induced NO production from macrophages [51]. gKO mice also are susceptible to *Mycobacterium tuberculosis* infection with heightened tissue necrosis and rapid death, although replacement of IFN-γ slowed the progression of the disease [52]. Work to further the investigation of IFN-γ on macrophage function revealed the importance of IFN-γ in decidual macrophage priming and found that spontaneous decidual production of IFN-γ can be detrimental to embryonic survival [53]. A recent study by Schaffer and colleagues found that gKO mice also have impaired NO synthesis in wound-derived macrophages thus resulting in unproductive wound healing [54].

CHAPTER III

PLACENTAL IMPROVEMENT AND REDUCED LIMB DEFECTS BY MATERNAL IFN-γ INJECTION IN METHYLNITROSOUREA-EXPOSED MICE

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ABSTRACT

BACKGROUND: Methylnitrosourea (MNU), an alkylating agent derived from creatinine metabolism, is cytotoxic, genotoxic, and mutagenic. Mid-gestational exposure to MNU leads to distal limb defects in mice. Previous studies have shown that nonspecific maternal immune stimulation protects against MNU-induced teratogenesis. A role for immune-mediated placental improvement in this effect remains uncertain.

METHODS: The immune system of timed-pregnant C57BL/6N and CD-1 mice was stimulated by gestation day (GD) 7 intraperitoneal (IP) injection with the cytokine, interferon-gamma (IFN-γ). A teratogenic dose of MNU was then administered by IP injection on the morning of GD 9 to disrupt distal limb formation. Fetal limb length, body length, digital deformities and placental integrity were evaluated on GD 14.

RESULTS:

The incidence of syndactyly, polydactyly, and interdigital webbing in MNU-exposed mice was decreased by maternal IFN-γ treatment. In C57BL/6N mice, these defects were reduced by 47%, 100%, and 63%, respectively; as compared to previous reports in CD-1 mice, by 39%, 71%, and 20% respectively. Administration of IFN-γ significantly diminished MNU-induced endothelial and trophoblast placental damage in both strains of mice.

CONCLUSIONS: These findings support a possible link between maternal immunity, placental integrity, and fetal distal limb development. Further, these results suggest that IFN-γ might act through placental improvement to indirectly protect against MNU-induced fetal limb malformations.

Key words: C57BL/6N mouse, CD-1 mouse, digit, interferon-gamma, limb, methylnitrosourea, placenta, teratogen

INTRODUCTION

It has been widely accepted that the immune systems of certain segments of the population diverge greatly from others, and these variations may be mimicked with the use of divergent murine strains in immunotoxicology studies. The inbred C57BL/6N mice were used in this study to simulate an immunologically-sensitive population and thereby determine if homozygosity within the strain impaired the teratogenic resilience. In a similar previous study by Prater, CD-1 mice were used to represent genetic and immunological hybrid vigor [35]. Immunologically, Toapanta and Ross found that outbred mice elicited more enhanced responses to antigenic complement compared to inbred mice [55]. It has also been found that CD-1 male mice were less susceptible to estrogenic compounds than inbred strains [56, 57]. These studies led us to question if we could expect a greater number of birth defects and less maternal immune protection in the strain (C57BL/6N) as opposed to the stock (CD-1) used in the 2004 study.

Alkylation lesions caused by MNU involve oxygen and nitrogen molecules of DNA, RNA or functional proteins and may lead to altered regulation of cell cycle or altered differentiation and proliferation of cells [22]. Gestational administration of MNU has been associated with birth defects by alkylation-related genotoxicity, increased production of reactive oxygen species (ROS), mitochondrial damage, or dysregulated apoptosis.

Administration of MNU to pregnant mice causes fetal ectrodactyly, microdactyly, syndactyly, polydactyly, and interdigital webbing [34]. Previous studies have shown that maternal immune stimulation protects against fetal teratogenesis, including distal limb

defects caused by MNU or urethane [58]. However, the mechanisms through which such protection is mediated are not yet understood. Cytokines of maternal immune system or placental origin, including colony stimulating factor-1 (CSF-1), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage- colony stimulating factor (GM-CSF), tumor growth factor-beta (TGF-β), interferon-alpha (IFN-α), and IFN-γ cross the placenta and have regulatory activity in the fetus, thus have been proposed as potential mediators of improved birth outcome [59, 60]. IFN-γ was chosen as the immune stimulant due to its wide range of immunological effects. IFN-γ is essential for the function of several components of the murine immune system including macrophages, natural killer (NK) cells, major histocompatability complex (MHC) antigens and T-cell activity [61]. In a study by Nomura et al., live activated macrophages with lysosomal enzymes were suggested to be effector cells that suppressed maldevelopment [62]. IFN-γ has also been shown to modulate tryptophan catabolism and thereby promote indoleamine 2,3-dioxygenase (IDO) expression preventing T-cell mediated allograft rejection [47, 63]. Punareewattana et al. found that the aforementioned treatments had the same phenotypic reduction of diabetic embryopathy but differed in microarray gene expression, suggesting that different immunomodulating agents may alter various pathways in embryonic development, but with a common outcome of fetal developmental protection against teratogenesis [64].

Alternately, maternal immune stimulation in urethane-exposed pregnant mice reduced placental damage and partially overcame a down-regulating effect of urethane on placental growth factor and cell cycle gene expression [41]. These authors therefore

suggested that placental improvement might in part explain decreased cleft palate and distal limb defects caused by immune stimulation in urethane-exposed pregnant mice. No correlation between placental function and reduced neural tube defects was detected, however, in diabetic mice after maternal immune stimulation [64]. These different results could in part relate to different teratogenic effects on placenta, i.e., urethane is cytotoxic and non-specifically targets placenta as a rapidly proliferating tissue, whereas diabetes induces placentomegaly [65]. MNU is cytotoxic and produces similar distal limb defects as urethane, therefore the present studies examined placental integrity in MNU-exposed pregnant mice with or without maternal immune stimulation, to determine if a correlation existed between placental changes and reduced birth defects.

MATERIALS AND METHODS

Mice

Six-week-old male and female C57BL/6N and CD-1 mice were obtained from Charles River Laboratories (Portage, MI) to be used as breeding animals for the experiment. Mice were acclimated for at least one week at 22.0 ± 1°C with 40-60% humidity and a 12/12 hour light/dark cycle. The mice were provided with food (2018 Teklad Global 18% Protein Rodent Diet, Harlan Teklad, Madison, WI) and fresh water ad libitum. Overnight breeding was conducted with a 1:1 male to female ratio. Every 12 hours females were checked for vaginal mucous plugs as an indication of breeding. Mice showing presence of a plug were designated as GD 0 and the male and female were

separated. Mice in all experiments were sacrificed on GD 14 by cervical dislocation. The Virginia Tech Institutional Animal Care and Use Committee reviewed and approved all experiments prior to their initiation. Institutional guidelines were adhered to in the treatment of all animals.

Experimental Groups

For both C57BL/6N and CD-1 mice, one of four treatments: negative control (vehicle), positive control (MNU), IFN-γ, or MNU + IFN-γ was assigned to dams in a generalized, randomized complete block design. Negative control mice received an IP injection of 100 µL Dulbecco's phosphate buffered saline (PBS), the vehicle used for MNU and IFN-γ. Positive control mice received an IP injection of 20 mg/kg MNU (Sigma-Aldrich Corp. St. Louis, MO) in 100 µL of PBS, on the morning of GD 9. This dose level and dosing schedule has been shown to induce distal limb defects in mice [62]. IFN-γ (Sigma-Aldrich; 1.0×10^3 IU in 100 µL of PBS, Sigma-Aldrich) was administered by IP injection in a single dose on GD 7. In previous experiments, IFN-γ given on GD 7 reduced the frequency of distal limb defects caused by GD9 MNU in outbred CD-1 mice [35].

Fetal limb defect and limb length determinations

Morphological deformities in the limbs and digits including syndactyly, oligodactyly, polydactyly, webbing and clubbing were quantified using an Olympus SZX7 stereomicroscope (Olympus Europa GmbH, Hamburg, Germany). Measurement of

limb length was performed using the same Olympus SZX7 stereomicroscope, with a DBX microscope to video camera coupler for direct image projection (Diagnostic Instruments, Inc., Sterling Hts., MI), and a Scion digital camera to acquire the image (Scion CFW-1310C 1394, Scion Corporation, Frederick, MA). The image was captured on the computer via a Scion firewire camera image acquisition application (version 1.0, Scion Corporation, Frederick, MA). Actual limb length measurements were obtained by converting the number of pixels in an image of the limbs to millimeters using Image J software, Version 1.30 from the National Institutes of Health (Bethesda, MA). Distal limb length measurements of the forelimbs were made from the olecranon tuber (point of elbow) to the tip of the longest distal phalanx. The hind limbs were measured from the calconeal tuber (heel bone) to the tip of the longest distal phalanx.

Histopathology

The numbers of fetuses and resorptions per dam were recorded and the fetuses and placentas were weighed on a top-loading balance (Accu-622, Fisher Scientific, Pittsburgh, PA). Resorptions were defined as small, dense, discolored spherical masses of tissue within the uterine tubes that did not contain grossly recognizable fetoplacental structures but histologically contained remnants of such [35]. The tissues were placed in 10% neutral buffered formalin for 18 hours and then transferred into 70% ethanol. The placentas were transected perpendicular to the long axis of the disc and stained with hematoxylin-eosin for morphological analysis under light microscopy. This plane of section allowed observation of the following layers, from maternal to fetal side:

myometrium, trophoblast giant cells and syncitiotrophoblasts (involved in production of growth factors and regulation of placental development), glycogen-rich trophoblasts (responsible for energy storage), and the highly vascular labyrinth layer (which is the site of maternal-fetal circulation exchange). Ten 400x fields of labyrinthine placenta from different dams in each treatment group were observed for frequency of necrotic endothelial cells as compared to controls. Similarly, ten 1000x fields of syncitiotrophoblast in each group were evaluated for the number of viable trophoblasts, as subjective observation suggested that trophoblast layers were thinner and somewhat devoid of viable cells following maternal exposure to MNU.

Statistics

One-way ANOVA and Tukey-Kramer HSD were used to evaluate crown to rump length and placental cell count data. ANOVA was used to compare means for the placental and fetal weights and for limb length interactions of MNU with IFN-γ with the MIXED procedure of the SAS System (Version 9.12, SAS Institute Inc., Cary, NC). The factorial treatment structure was analyzed with contrasts constructed to test for interaction effects of MNU with IFN-γ. Significant interactions were further investigated using tests of simple main effects. Model adequacy was assessed using standardized residual plots. Data described as different in this report implies statistically different, $p<0.05$.

Because of the relative infrequency of the birth defects in the fetuses, categorical data were utilized from the numerical incidence of findings instead of statistical analysis in order to avoid erroneous conclusions.

RESULTS

Fourteen litters, including 117 fetuses were examined in the control group. MNU-only-exposed dams yielded 94 pups in 18 litters. In the IFN-γ treatment group, 7 litters including 51 fetuses were evaluated, and lastly in the combined treatment group of MNU + IFN-γ, 9 litters including 45 fetuses were collected.

Placental and fetal weights, fetal resorptions

Mean placental weight from control dams was greater than MNU-exposed dams (C57BL/6N: 121.4 ± 1.2 mg vs. 90.3 ± 1.1 mg; CD-1: 112.0 ± 0.0 mg vs. 93.0 ± 0.0 mg; Fig. 1). Maternal IFN-γ injection in C57BL/6N mice on GD 7 caused a small decrease in placental weight relative to control, to 116.8 ± 1.5 mg. In CD-1 mice, IFN-γ injection caused an increase in weight to 126.0 ± 0.0 mg. A similar placental weight increase occurred in diabetic CD-1 mice after maternal INF-γ injection [66]. Mean placental weight in C57BL/6N mice that received both MNU and IFN-γ was increased relative to MNU only, to 113.4 ± 1.6 mg, indicating IFN-γ protected against MNU-induced decreased placental weight in the immunosensitive strain. In the CD-1 mice, no significant increase in placental weight occurred in MNU-exposed dams as a result of IFN-γtreatment.

Mean fetal weight from control dams was greater than MNU-exposed dams (C57BL/6N: 265.4 ± 2.0 mg vs. 207.9 ± 1.8 mg; CD-1: 302.0 ± 0.0 mg vs 237.0 ± 0.0 mg; Fig. 2). Similar to placental weight, IFN-γ alone reduced fetal weight relative to control in C57BL/6N mice to 238.5 ± 2.5 mg, and increased fetal weight to 315.0 ± 0.0 mg in CD-1 mice. The mean fetal weight in mice that received both MNU and IFN-γ was 210.8 ± 2.7 mg (C57BL/6N) and 260.0 ± 0.0 mg (CD-1), neither different from MNU alone.

The number of fetal resorptions in the C57BL/6N mice ranged by treatment from a low of 6% in the MNU exposure group to a high of 9% in the MNU + IFN-γ group. This low level of resorptions was also displayed in the CD-1 mice. These levels fell within the expected range for these strains of mice, at 10% or below [67], and were not significantly different between groups.

Fetal limb length, body length, and defects

IFN-γ administration did not affect fetal forelimb or hindlimb length relative to control in the C57BL/6N or CD-1 mice (Table 1). MNU decreased both forelimb and hindlimb lengths in both strains of mice. The combination of MNU + IFN-γ caused either increased shortening of fetal left limbs or trends toward shortening in both strains of mice, as compared to MNU only. In the measurement of mean body length, IFN-γ-treated fetuses were not different from control (Table 1). MNU treatment reduced the absolute body length of the fetuses to less than control or IFN-γ. Applied together, an approximate additive decrease in body length occurred from MNU + IFN- γ treatment.

Fetal limb and body length measurements generally appeared to decrease in a proportionate manner after MNU or MNU + IFN-γ. However, in C57BL/6N mice, when ratios of limb length to body length were calculated, the forelimbs of MNU and MNU + IFN-γ treated groups were found to be disproportionately shorter than would be expected from simple reduced fetal size that also occurred in these groups (Table 2). The left hind limbs of the MNU and IFN-γ groups, after correction for body length reduction, were similarly disproportionately shorter than control, while the MNU + IFN-γ group showed a further decrease in corrected limb length. In the CD-1 fetuses, IFN-γ caused disproportionate shortening in the forelimbs and left hindlimb as compared to control (Table 2).

Pictorial examples of distal limb defects caused by MNU in C57BL/6N mice are shown in Figure 3 and are representative of those also seen in CD-1 mice; the defects are also numerically represented in Table 2. In agreement with previous reports in CD-1 mice [35], maternal immune stimulation reduced occurrence of these defects. In C57BL/6N mice, syndactyly, polydactyly, and interdigital webbing were reduced by 47%, 100%, and 63%, respectively, by IFN-γ injection (Table 3). In the CD-1 mice these same defects were reduced by 39%, 71%, and 20% respectively [35].

Placental Histopathology

Hematoxylin and eosin sections of GD 14 placentas were evaluated under light microscopy for alterations in placental architecture across treatment groups. Placentas from MNU-treated dams displayed multifocal areas of necrosis that predominantly

targeted endothelium in the labyrinthine layer, with karyolysis and pyknosis, cellular fragmentation, hypereosinophilia, and collapse of vascular patency (Fig. 4c). Areas of necrotic trophoblasts were also seen in the syncitial layer and the trophoblast giant cell layer with mild suppurative inflammation and dystrophic mineralization. Lesions were rarely visualized in the control placentas, and included occasional necrotic or pyknotic trophoblasts and endothelial cells (Fig. 4a). These observations in controls were considered within the range of normal for the placenta, a rapidly dividing and dynamic tissue at this point during gestation. Placental samples from IFN-γ treated dams displayed occasional mild suppurative inflammatory infiltrates superficial to the trophoblast giant cell layer, with rare evidence of necrotic cellular debris in the labyrinthine and trophoblast layers (Fig. 4b). The placentas from MNU + IFN-γ treated dams displayed a hybrid of these lesions, with occasional neutrophilic infiltration at the level of the trophoblast giant cell layer and scattered focal areas of necrosis in the labyrinthine layer (Fig. 4d).

An average of 25.2 ± 1.2 necrotic cells per 400x magnification field were observed in the labyrinthine layer of the MNU treated placenta, as compared to 0.3 ± 1.2 per field in controls, 0.5 ± 1.2 in the IFN-γ group, and 3.6 ± 1.2 in the MNU + IFN-γ placentas (Fig. 5). The average number of viable trophoblasts per field in the syncitiotrophoblast layer of control and treatment groups was evaluated under 1000x magnification. In MNU-treated mice, there was an average of 33.4 ± 2.1 viable trophoblasts, as compared to 50.1 ± 2.1 in controls, 48.9 ± 2.1 in IFN-γ, and 38.9 ± 2.1 in MNU + IFN-γ (Fig. 6).

Placental histopathological findings in CD-1 mice differed in magnitude but were otherwise similar in pattern to C57BL/6N mice. Sections showed an average of 12.3 ± 0.9 necrotic endothelial cells in the MNU-treated CD-1 mice, as compared to 0.6 ± 0.9 in controls, 3.6 ± 0.9 in IFN-γ, and 7.7 ± 0.9 in MNU + IFN-γ̃ MNU decreased placental trophoblasts, with only 25.2 ± 1.4 viable cells seen following MNU treatment, as compared to 51.3 ± 1.4 trophoblasts in controls, 48.0 ± 1.4 in IFN-γ, and 38.1 ± 1.4 in MNU + IFN-γ.

DISCUSSION

Bochert et al. proposed that DNA alkylation in embryonic tissues was the primary cause of birth defects induced by alkylating agents[23]. An additional contributing role for reactive oxygen species (ROS) was later suggested by the observation of diminished placental damage and reduced teratogensis in MNU-exposed mice, after maternal dietary antioxidant exposure [3]. These authors noted that maternal IFN-γ supplementation reduced a variety of teratogen-induced defects in mice (for a review, see [60]); and that antioxidant therapy increases circulating IFN-γ levels [68]; thus suggested increased IFN-γ may in part reduce fetal defects in MNU-treated pregnant mice through beneficial effects on placenta.

Immune stimulation with either Freund's complete adjuvant (FCA) or IFN-γ reduced ultrastructural damage to the placenta caused by urethane, a non-specific cytotoxic agent [41]. Placental genes for Th2-type cytokines (e.g., IL-4; IL-10; IL-13) were up-regulated by both methods of immune stimulation in the urethane-treated mice,

while urethane alone shifted placental cytokine gene expression profile toward Th1 cytokines including IFN-γ and IL-2. The authors noted a correlation between improved placental structure and function, and decreased fetal defects caused by immune stimulation. And in contrast to Sharova's study demonstrating a muted response of CD-1 mice to the protective effects of immune stimulation, the results of the present study further noted a greater improvement in birth defect outcome in immune-stimulated, teratogen-exposed CD-1 mice as compared to similarly treated immune sensitive C57BL/6N mice. The latter may suggest protective mechanisms related to immunologic response differences between these strains.

Loke and King described the recruitment of bone-marrow derived macrophages and other large granulated lymphocytes to the uterus during early pregnancy [69]. The potential activity of regulatory cytokines derived from uterine leukocytes, including IFN-γ in maintenance of the placenta was also suggested [70]. Antigen-specific T and B lymphocytes are largely absent at the materno-fetal interface of species with hemochorial placentation, including rodents and humans. In contrast, non-antigen specific macrophages and NK cells are abundant at this location. Recent studies indicate that these immune cells have major placenta-associated functions that include stimulation of maternal vasculature growth to placenta, supply of growth factors to placenta, and promotion of placental trophoblast differentiation [71]. Savion et al. reported that maternal intrauterine injection with the cytokine, GM-CSF caused an increase in uterine macrophages in cyclophosphamide-exposed mice, and decreased fetal craniofacial defects caused by this therapeutic alkylating agent [72]. Like GM-CSF, IFN-γ is a potent

macrophage-stimulating factor [73]. Beneficial effects to placenta in rodents exposed to alkylating agents, as a result of maternal immune stimulation, have not been previously reported or to our knowledge examined. Placental weight was differentially affected in the present two strains of mice. MNU decreased weight of the placenta in both strains, an effect overcome by maternal IFN-γ injection in the CD-1 mice but not the C57BL/6N mice. Punareewattana and colleagues noted similar increased placental weights in pregnant diabetic CD-1 mice after maternal IFN-γ injection, but not after maternal FCA or GM-CSF injection [66]. Together, these studies suggest that placental improvement may be a contributory, but not a primary factor in protection against teratogenesis, and that there may be other mechanisms by which fetal development is altered by midgestational maternal immune stimulation. Diabetes-induced neural tube defects in the mice were reduced, equivalently, by the three different forms of maternal immune stimulation, suggesting increased placental weight may not have been related to improved morphologic outcome. Similarly, the present mice showed approximately equal immune protection against distal limb defects, but with placental weight improvement only in the C57BL/6N mice.

The histopathologic examination of placentas after MNU showed predictable damage, given the proliferating nature of placenta and the cytotoxicity of MNU. Placentas from animals that also received IFN-γ presented with clear signs of reduced damage under light microscopy. Necrotic cells were therefore enumerated to partially quantify the different level of placental damage. These cells were abundant in both strains of mice after MNU, and significantly diminished in mice that also received IFN-γ.

Placental trophoblast number displayed a pattern opposite to necrotic cells, being diminished by MNU alone but increased after MNU + IFN-γ. This outcome may relate in part to promotion of placental trophoblast differentiation by cytokines produced by local leukocytes [71], which are increased by maternal IFN-γ injection [66].

Fetal distal limb (manus/pes or foot) defects were diminished in MNU-treated mice after IFN-γ, similar to several previous reports [35, 62, 74]. The present mice showed disproportionate distal limb shortening after MNU, i.e., the limbs were still shorter than control after correction for reduced fetal size in MNU dams. Unexpectedly, IFN-γ alone caused limb shortening, and, when co-administered with MNU, worsened the limb shortening effect of MNU. Nonetheless, digital defects were decreased by IFN-γ. This outcome suggests that limb lengthening and distal limb establishment may be independent events, regulated by different genetic or post-gene expression mechanisms that differentially respond to IFN-γ. This observation may support the hypothesis of Sharova et al., that IFN-γ or cytokines of maternal origin induced by IFN-γ and capable of crossing the placenta (e.g., TGF-β), directly affect fetal development [75]. In this regard, TGF- β is an important regulator of timed waves of cellular differentiation during digit formation [76]. Alternately, maternal IFN-γ may increase cytokines of placental origin (e.g., CSF-1) that cross placenta and are equally important in fetal development [77].

In summary, the present results show clear reduction of MNU-induced placental damage by maternal immune stimulation with IFN-γ. This observation supports the hypothesis that defect reduction in immune stimulated mice may at least in part be a

direct consequence of improved placental function. This hypothesis clearly has attractive features, given the remarkably broad-spectrum nature of reduced defects seen in immune stimulated mice following exposure to chemically and physically diverse teratogens (reviewed by [78]). However, a mechanistic link between placental improvement and reduced birth defects remains to be identified. Further, the present results in no way rule out a role of transplacental mediators of maternal origin in reduced birth defects.

CHAPTER IV

Altered Placental Cytokines in JakStat Signaling Pathway due to MNU-Exposure

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Abstract:

Maternal immune stimulation with interferon-gamma (IFN-γ) has previously been shown to reduce birth defects in the offspring of dams given the teratogen methylnitrosourea (MNU). Digital malformations are reduced and fetal limb and body length are improved. The placenta is also protected from MNU-induced necrosis by trophoblast and endothelial cell protection when IFN-γ is given concurrently. Protein array studies have elucidated the Jak-Stat pathway as being important in regulating the cytokine environment at the feto-maternal interface. The present work utilized pathway focused gene microarrays from gestation day (GD) 12 C57BL/6N mouse placentas to determine differential gene expression as a result of IFN-γ administration. We found that hepatocyte growth factor (HGF), insulin growth factor 2 (Igf2), interleukin 1 beta (IL1β), fibroblast growth factor 3 (Fgf3), and interferon-related developmental factor 1 (Ifrd1) all showed significant alterations due to MNU treatment and pretreatment with IFN-γ. The Jak-Stat pathway is associated with Hgf, Igf2, and IL1β signaling and therefore this study provides another mechanistic link in the investigation of birth defect amelioration by way of maternal immune stimulation.

Introduction:

Each year, 7.9 million babies are born with birth defects. Seventy percent of those could be prevented, ameliorated, or repaired; yet 3.2 million children still die by the age of three (March of Dimes Global Report 2006). Many factors, including genetics, lifestyle, maternal age, and prenatal care and nutrition contribute to the occurrence of birth defects. Vitamins, minerals, immune modulators, exercise, and diet regimens have been employed to help decrease fetal defects and preterm birth. A key site in the maintenance of a viable pregnancy is the feto-maternal interface (uterus, chorion, decidua, spiral arteries, placenta). Proper placental development, as well as adequate uterine architecture, ensures that the exchange of nutrients, gases, and immunological signals is achieved between mother and conceptus. Immune stimulants have been shown to reduce fetal birth defects and placental maldevelopment. The mechanism of the amelioration of these effects due to immune stimulation is unknown.

IFN-γ is a non-specific immune stimulant that can be harmful or helpful to pregnancy, depending on concentration and time of administration. Chaouat et al. found that it is abortifacient at high doses in synergy with TNFα [42]. However, Ashkar & Croy identified a crucial spike of IFN-γ at implantation and determined that it is necessary for local vascularization at the fetomaternal interface [43-46]. Work by Munn & Mellor found that IFN-γ induces antigen presenting cell expression of indoleamine 2,3 deoxygenase, which catabolizes tryptophan and results in decreased T-cell proliferation [47-49]. IFN-γ has also been previously shown to ameliorate teratogenic effects of methylnitrosourea (MNU), a potent mutagen, carcinogen, and teratogen that exerts its

detrimental effects via DNA alkylation. Our lab has shown that the distal limb malformations syndactyly, polydactyly, and webbing are reduced by 47%, 100%, and 63% respectively when IFN-γ is given 2 days prior to MNU administration [4].

We have also observed that IFN-γ works at the placental level to protect against MNU-induced damage. Trophoblast loss and associated cytokine alterations occur in GD14 placenta following GD9 MNU exposure, showing that fetal-maternal communication can be hindered due to MNU. In the labyrinthine layer of the placenta, multifocal fibrinous necrosis of endothelial cells can be observed due to MNU administration, however IFN-γ almost completely protected the trophoblast and endothelial cells when given to the dam as an immune stimulant [4].

These reduced birth defects and improved placental architecture as a result of IFN-γ administration have led us to believe that immune modulation promotes proper placental development and thereby reduces fetal maldevelopment.

Protein array data from analysis of GD14 placentas in our lab suggested the janus activating kinase-signal trandsducer and activator of transcription (Jak-Stat) and nuclear factor kappa B (NFκB) pathways as being key regulators of the mechanisms promoting birth defect reduction through maternal immune stimulation and with the antioxidant butylated hydroxytoluene (BHT) [79]. The Jak-Stat pathway explained 40% of the gene expression changes observed in the study, by principal component analysis [79]. We continued this investigation at the genomic level by employing pathway-focused gene microarrays to determine the differential gene expression in the placental tissue from GD12 fetuses. These mouse-cytokine focused arrays provided a systematic method by

which we could determine the genes and cytokines present and relevant in this model and allowed us to evade investigating a gene based only upon our knowledge of its biological role.

The genes hepatocyte growth factor (Hgf), insulin growth factor 2 (Igf2), interleukin 1 beta (IL1β), fibroblast growth factor 3 (Fgf3), and interferon-related developmental factor 1 (Ifrd1) all showed significant alterations due to MNU treatment and when IFN- γ was given concurrently. The Jak-Stat pathway has been associated with Hgf, Igf2, and IL1β signaling by mediating the cellular responses to the growth factors and cytokine. Fitzgerald and colleagues suggested that the Jak-Stat pathway crosses the receptor-associated tyrosine kinase-mitogen-activated protein kinase (RTK-MAPK) pathway and has important effects during trophoblast invasion of the placenta [80]. The present investigation provides direct evidence of the genes differentially regulated in GD12 placenta of C57BL/6N mice when IFN-γ is given to reduce birth defects. We also investigate the mediation of the Jak-Stat pathway, providing important information in the ongoing research to uncover the mechanisms behind birth defect reduction through maternal immune stimulation.

Materials & Methods:

Mice:

This investigation utilized a mouse model with the teratogen MNU to induce birth defects. MNU is also a potent carcinogen and mutagen through its action as an alkylating

agent. Depending on the gestational day of administration, MNU can cause digital limb defects, placental damage, craniofacial defects, retinal malformation, or fetal resorptions.

Six-week-old male and female C57BL/6N mice were obtained from Charles River Laboratories (Portage, MI) to be used as breeding animals for the experiment. Mice were acclimated for at least 1 week at 22.0 +/-1°C with 40–60% humidity and a 12/12 hr light/dark cycle. The mice were provided with food (2018 Teklad Global 18% Protein Rodent Diet; Harlan Teklad, Madison,WI) and fresh water ad libitum. Overnight breeding was conducted with a 1:1 male-to-female ratio. Every 12 hr, females were checked for vaginal mucous plugs as an indication of breeding. Mice showing the presence of a plug were designated as GD 0, and the male and female mice were separated. The Virginia Tech Institutional Animal Care and Use Committee reviewed and approved all experiments prior to their initiation. Institutional guidelines were adhered to in the treatment of all animals.

Dams were assigned to treatment groups in a generalized, randomized, complete block design. Treatment groups consisted of a negative control (vehicle only), MNU-treated, IFN-γ-treated, and MNU+IFN- γ treated mice. Negative control mice received an intraperitoneal (IP) injection of 100 µl of Dulbecco's PBS, the vehicle used for both MNU and IFN-γ. Positive control mice received an IP injection of 20 mg/kg MNU (Sigma-Aldrich, St. Louis, MO) in 100 µl of PBS on the morning of GD 9. IFN- γ (Sigma-Aldrich; 1.0 x 103 IU in 100 µl of PBS) was administered by IP injection in a single dose on GD 7. The dose and timing of MNU administration has been shown to induce distal limb defects in mice [62]. In previous experiments, IFN- γ given on GD 7

reduced the frequency of distal limb defects caused by GD 9 MNU in outbred CD-1 mice [35].

Tissue Collection and Total RNA Isolation:

Mice in all experiments were euthanized on GD 12 by cervical dislocation. Uteri were excised and fetuses were separated from placentas. The placentas were weighed on an Adventurer™ balance (Ohaus, Pine Brook, NJ) and placed in RNAlater™ (Ambion, Austin, TX) to prevent mRNA degradation. The placentas were stored at -20°C until RNA isolation using RNeasy® Mini Kits (Qiagen, Valencia, CA). 10-30 mg of placental tissue were homogenized with a rotor-stator tissue homogenizer (Tissue Tearor, Biospec Products Inc, Bartlesville, OK) in guanidine-isothiocyanate buffer and 2-mercapthoethanol (Sigma-Aldrich, St. Louis, MO). The lysate was passed through the gDNA eliminator spin column for genomic DNA removal. The flow-through was then mixed with ethanol and applied to the RNeasy spin column for RNA purification. The RNA was eluted with 30 uL Rnase free water and immediately tested for concentration and quality using a BioPhotometer spectrophotometer (Eppendorf, Hamburg, Germany). The minimum 260/280 nm ultraviolet (UV) absorbance acceptable was set to be 1.8. Isolated total RNA was stored at -80°C until cDNA synthesis.

cDNA Synthesis:

3 μg RNA were used for cDNA synthesis using SuperArray's AmpoLabeling-LPR kit (Frederick, MD). Biotin-16-dUTP (Roche, Nutley, NJ) was used to label the

probe and the linear polymerase reaction involved 30 cycles of 85°C, 1 min; 50°C, 1 min; 72°C, 1 min using a Techne Techgene Thermal cycler (Burlington, NJ). The probe was denatured at 94°C for 2 minutes (Isotemp heating block, Thermo Fisher Scientific, Waltham, MA) and quickly cooled on ice. The probe was then applied to the prehybridized microarray.

Microarrays:

GEArray Q Series Mouse Common Cytokines gene arrays were used in this study because of the assortment of functional genes of particular interest for this study (SuperArray, Frederick, MD). The arrays contained 96 genes, from functional groups such as bone morphogenic proteins, colony stimulating factors, fibroblast growth factors, interferons, insulin-like growth factors, interleukins, platelet-derived growth factors, transforming growth factors, tumor necrosis factors, and vascular endothelial growth factors. The housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GapDH), cyclophlinA, ribosomal protein L13A (RPL13A), and beta-actin were also contained on the arrays.

The microarrays were prehybridized by application of a prehybridization solution containing heat-denatured sheared salmon sperm DNA (SuperArray) for approximately 2 hours at 60°C (Isotemp Hybridization Oven, Thermo Fisher Scientific, Waltham, MA). The entire volume of the denatured cDNA probe was added to prehybridization solution and allowed to hybridize to the arrays in their hybridization tube overnight at 60°C with constant rotation. The arrays were washed with increasing stringency the following

morning. Bound probe was detected using an alkaline phosphatase-conjugated streptavidin and chemiluminescence (SuperArray). The arrays were placed in syran wrap for exposure to X-ray film (Kodak BioMax XAR Film, New Haven, CT). Multiple exposures were taken, ranging from 10 seconds to 2 minutes, depending on signal intensity and saturation of the housekeeping genes.

Microarray Analysis:

Array images were scanned (Canoscan 9900F, Canon, Tokyo, Japan) and converted to TIF files for importation into the GEArray Analysis Suite (SuperArray). Within the analysis suite, a template containing the 8X14 grid of tetra-spots was applied on top of the array and arranged to match the exact representation of spots on each array. Data were extracted from the software as the average density of each tetra-spot, with global background correction and with no normalization. These data from each array were individually exported for statistical analysis. After obtaining the top 10 most differentially expressed genes from the control versus MNU treatment comparison, those genes were referenced back to the analysis suite software to determine if the collective (all samples within the treatment group) expression of the gene of interest was considered present. SuperArray defined absent genes as those spots with less than the mean value of the local backgrounds of the lower 75 percentile of all non-bleeding spots.

For validation of microarray data, real-time reverse transcriptase polymerase chain reaction (RT PCR) is necessary to confirm differential gene expression of the

samples using specific primers for each gene of interest. These data are not included in this document.

Statistics:

Background adjusted raw probe intensities were extracted from SuperArray image processing software and exported into the open source Bioconductor (http://www.bioconductor.org/) [81] collection of packages for further processing and statistical analysis. Data were normalized [82], and a generalized logarithmic transformation applied using the variance stabilization (VSN) package [83, 84]. To obtain differentially expressed genes, the Bioconductor package limma was used to fit a linear model to the data (with treatments as factors), followed by pair wise contrasts [85]. For every contrast, the top 10 most differentially expressed genes were selected using empirical Bayes moderated t-statistic while controlling for the false discovery rate. False discovery rate and level of statistical significance were set at 5%.

Results:

Microarrays:

Idiopathically, the gene expression fold changes in the microarrays were not greater than a 2-fold magnitude. Hgf, Fgf3, Igf2, IL1β, and Ifrd1 were all reduced in expression after MNU administration in comparison to control (Figure 7). IFN-γ resulted in a slight, numeric increase in gene expression in all the genes except for Ifrd1.

Statistical analysis indicated that IFN-γ given prior to the teratogen did not improve the decreased expression of Hgf, Fgf3, Ifrd1 and Igf2 due to MNU alone. However, IFN-γ in addition to MNU did cause IL1β expression to increase to even greater expression than observed in the control or IFN-gamma only treatment groups.

Discussion:

The long-term goal of our laboratory is to elucidate the mechanisms involved in birth defect reduction due to maternal immune stimulation. Understanding the mechanism(s) by which the immune system modulates placental development and protects the fetus from teratogenesis is important to the field of reproductive biology and has the potential to reduce the prevalence of birth defects worldwide. We believe that many mechanisms are at work, be it independently or synergistically, to promote the proper development of the placenta as well as the fetus. Due to the role of the placenta in transport of nutrients and gases, this organ is likely the primary modulator of the pathways influencing development. Although, the uterus, mesometrial lymphoid aggregate of pregnancy, and deciduas are also contributing tissues of regulation and would likely demonstrate varied gene expression than observed in the placenta. On a similar note, the time point in gestation at which the tissues are collected will yield varying results based on the increased and decreased levels of the cytokines at different points of development. GD12 placenta was chosen for gene analysis in order to attempt

to capture the gene expression of the cytokines that would influence the phenotypic differentiation of the limbs, collected at GD14 (the time of appendicular growth).

The genes we chose to examine of those that the microarrays elucidated as being significantly differentially expressed in GD12 placenta were IL1β, Hgf, Igf2, Fgf3, and Ifrd1. Providing correlation between the first three is the Jak-Stat pathway, which works by modulating cellular responses to cytokines and growth factors. Cytokine or growth-factor binding to cognate cell-surface receptors activates the Jaks to phosphorylate tyrosine residues on the receptor and attract Stats to the receptor where they bind and will become tyrosine or serine phosphorylated. The Stats then dimerize as hetero- or homodimers and translocate to the nucleus. In the nucleus they act on promoter-specific regions of their target genes to control transcription. An inhibitory feedback mechanism exists to reduce Stat-mediated signaling through the transcription of the suppressors of cytokine signaling (SOCS).

The most important Stat relating to this work is likely Stat3. Loss of function studies of Stat3 have revealed that it is necessary for embryogenesis [87, 88]. It has been localized to extraembryonic visceral endoderm at GD7.5 and is directly linked to trophoblast invasion, a necessary element in the building of the placental as a conduit of gas and nutrients between embryo and mother. Further study is needed to determine the disposition of Stat3 following teratogen exposure plus and minus immune stimulation in order to further elucidate the interaction of the Jak-Stat pathway and maternal immunity in placental and distal limb formation.

HGF

HGF is a secreted protein that acts as a mitogen, motogen, and morphogen through its receptor, c-Met. HGF mRNA is expressed in mesencymal cells of the villi, and c-Met is expressed in cytotrophoblast cells [89]. Paracrine signaling pathways of HGF/c-Met mediate proliferation and differentiation of epithelial cells, migration of the myogenic limb bud precursors to the limb bud [90], stimulation of endothelial cell growth [91], invasion of cytotrophoblasts in first and second trimester human placenta [92], and extension of villous branching in the chorioallantolic placenta [93]. These effects can occur via various signaling pathways including STAT, NF-kB, MAPK, PI3-kinase, phospholipase C, and Src [93-95].

Pathologically, Baykal et al. determined that deregulation of the interaction between HGF and c-Met could attribute to fetal growth restriction in intrauterine growth restricted (IUGR) fetuses[96]. Reduced levels of HGF lead to shallow spiral artery invasion and inadequate trophoblast invasion and are associated with pre-eclampsia [97]. HGF null embryos possess insufficient placental architecture and die before birth [98]. Specifically, these mutant mice have reduced labryinthine layers of the placenta with poorly developed vasculature. Uehara detected the abnormalities as early as GD 10.5, with death ensuing by GD 17.5 [98].

We previously identified an increased number of necrotic endothelial cells in the labyrinthine layer of GD12 placentas after MNU treatment [4] and therefore believe that HGF in these C57BL/6N mice may be decreased indirectly by the teratogen and result in the observed placental dysfunction and development.

IL1β

IL1β comes from the IL-1 cytokine superfamily; it acts extracellularly and is increased by estrogen and progesterone during pregnancy. This cytokine is necessary for pre-implantation and implantation, and Huleihel and colleagues observed increased levels in uterine artery [99]. IL1β expression is increased by the conceptus and released into the uterine lumen during initiation of rapid trophoblast invasion [100]. Endometrial stromal cells also increase in secretion of proinflammatory cytokines due to IL1β stimulation, and this mechanism has been attributed to the MAPK pathway [80]. Fitzgerald proposed the linkage of the Jak-Stat and MAPK pathways of being via direct interaction, a common binding site (Tyr759), phosphorylation of Stat3, or SOCS.

Another possible hypothesis relating to this cytokine is that uNK-derived IFN-γ maintains decidual viability and prevents birth defects by inducing placental IL1β and it's related cytokine, IL-18 expression. In IL-18 null mice, the concurrent loss of IL1β did not increase the rate of preterm birth [101]. Although IL-18 was not significantly altered in the GD12 placenta in the present study, it is possible that a different collection day could yield a results showing that IL-18 is differentially expressed and contributing the uNK differentiation and mediation of the feto-maternal interface. IL1β could also be activating the NFκB pathway via the IL1R and thus it can continue to promote the transcription and production of other pro-inflammatory cytokines. Voronov and colleagues suggested that IL1β mediated the immunostimulatory and inflammatory pathogenesis surrounding systemic lupus erythematosus (SLE) based off of their loss of

function study where the knock out of IL1β alone led to a non-severe disease state of systemic lupus erythematosus [102].

Igf2

Igf2 is the major regulator of growth in utero. Produced by the fetus after GD 10.5, Igf2 mRNA is found in placental tissues from GD 5.5. Igf2-null and placenta-specific-Igf2-null mice have reduced placental and fetal size [103-106]. Specifically, Gardner found that Igf2 deficiency in the trophoblast reduced mean placental weight by 21%, whereas ICM deficiency of Igf2 reduced mean placental weight by 14% [104]. The same study also concluded that fluid transport from mother to fetus or fetal retention of water was impaired due to Igf2 deficiency. Overexpression of Igf2 results in fetal overgrowth [107, 108]. Because insulin-like growth factors induce vascular endothelial growth factor (VEGF) mRNA expression, it is possible that differential Igf2 levels indirectly mediate the angiogenic and vascular permeability effects of VEGF at the feto-maternal interface. The effects of Igf2 are mediated by MAPK and then Stat3 in the Jak-Stat pathway.

Fgf3

Also known as Int2, Fgf3 was initially discovered in association with mouse mammary carcinomas, however, plays an important role in development. Fgf3 is active in the development of the retina, inner ear, and is found in presumptual teeth mesenchyme [109-111]. Jakobovits found Fgf3 in endodermal cells prior to GD 7.5

[110]. However, Rappolee reported that Fgf3 was not present in preimplantation embryos [112] and further microarray and Q-PCR analysis by Zhong and colleagues confirmed the absence of Fgf3 at GD 1.5 and 3.5 [113]. Mansour found that the targeted disruption of Fgf3 in fetal mice resulted in short, curled tails and death before or immediately following birth [114]. His research suggested that the tail defects were due to the disruption of Fgf3 in the primitive streak and tail bud. He also found that mutant mice that survived past birth had inner ear abnormalities. Mutations in the Fgf3 tyrosine kinase repeptor, Fgfr3, can result in thanatophoric dysplasia, hypochodrophlasia, achondroplasia or craniosynostosis syndromes. This is believed to be due to cysteine residue formation leading to mutant monomers that enter the nucleus and interfere with chondrocyte development. Fgf3 signaling can occur via various receptors such as cystein-rich receptors, high-affinity cell surface receptors (FGFR) with intrinsic tyrosine kinase activity, lower-affinity heparan sulfate-containing proteoglycan receptors, and by directly entering the nucleus [115-117].

Ifrd1

Interferon related developmental regulator 1, also known as Tis7 (tetradecanoyl phorbol acetate-induced sequence 7 gene) or PC4, is responsive to tetradecanoyl phorbol acetate, epidermal growth factor and fibroblast growth factor in vivo and in vitro [118-122]. Activation by c-Jun can result in transcriptional coregulation in epithelial cells [123, 124]. PC4-null mice are viable but infertile, possibly due to inefficient fertilization of the oocyte by the sperm [125].

Several possibilities exist for the discrepancies between the PCR and microarray data. Although the microarrays and primers were from the same vendor, the probe sequences on the arrays were different than those used to create optimal primers. For this reason, we cannot assume that the PCR and microarray data will be the same. We conclude that using an alternate array system could improve the concordance of the platforms used in this experiment. Splice variants due to altered patterns of exon splicing could also contribute to the discordance between the platforms, as many of these genes have published splice variants [126-130].

Overall, we are able to provide evidence that IL1β, HGF, Fgf3, Igf2, and Ifrd1 are actively influencing the cytokine and immune environment of the feto-maternal interface at GD12, which are likely contributing to altered appendicular development later in gestation. We have observed that IFN-γ administration with MNU does not influence these genes in a strong enough fashion to conclude that they are the main mediators of birth defect reduction at GD12, however we are able to conclude that the Jak-Stat pathway is the common and important link mediating the immunological and cytokine response, and that this pathway is likely a large part of the cytokine response that could be observed at other gestational timepoints. We expect that on the days surrounding GD12, these genes could be even more significantly altered, however, we do not expect to see a large differential expression of genes related to trophoblast and endothelial cell growth and differentiation since the placenta is nearly fully developed by GD 14.5 [131,

132]. Loss of function studies of these genes could provide more evidence of their role in immune-stimulant modulated birth defect reduction.

CHAPTER V

Conclusions

Descriptive research in the field of developmental immunotoxicology must be followed by mechanistic studies in order to really provide useful contributions to birth defect reduction. This work has provided both descriptive and mechanistic results. We determined that maternal treatment of IFN-γ reduced the incidence of syndactyly, polydactyly, and interdigital webbing in MNU-exposed mice. In C57BL/6N mice, these defects were reduced by 47%, 100%, and 63%, respectively; as compared to previous reports in CD-1 mice, by 39%, 71%, and 20% respectively [35]. Administration of IFN-γ also significantly diminished MNU-induced endothelial and trophoblast placental damage in both strains of mice. These findings support a possible link between maternal immunity, placental integrity, and fetal distal limb development. Further, these results suggest that IFN-γ might act through placental improvement to indirectly protect against MNU-induced fetal limb malformations (Figures 8 & 9).

From gene microarray studies of GD12 placenta, we found that hepatocyte growth factor (HGF), insulin growth factor 2 (Igf2), interleukin 1 beta (IL1β), fibroblast growth factor 3 (Fgf3), and interferon-related developmental factor 1 (Ifrd1) all showed significant alterations due to MNU treatment and when IFN-γ was given 2 days prior. The Jak-Stat pathway is associated with Hgf, Igf2, and IL1β signaling and therefore this study provides a mechanistic link in the investigation of birth defect amelioration by way of maternal immune stimulation.

Suggested Future Investigations

Tissue-specific loss of function studies could be performed in future studies to determine the relative importance of the genes afore-mentioned in birth defect reduction and placental protection. Teratogenesis studies with gKO mice and MNU should also be used to determine the baseline effects of MNU in the absence of IFN- γ's pleiotropic effects. Furthermore, examination of uNK cells and trophoblasts with immunohistochemistry could provide important data for determining what cell types are producing and being influenced by the genes of interest.

Future studies could also include an investigation of the uterine tissue from MNU and IFN- γ-treated dams. At the same time, uterine and placental tissue collection could encompass a more expansive timeframe in order to capture a broader cytokine environment and determine the effect of the genes of interest at various timepoints.

REFERENCES

1. Brambell, F.W., *The transmission of immunity from mother to young and the catabolism of immunoglobulins.* Lancet, 1966. 2(7473): p. 1087-93.
2. Carpenter, S.J. and V.H. Ferm, *Uptake and storage of Thorotrast by the rodent yolk sac placenta: an electron microscopic study.* Am J Anat, 1969. 125(4): p. 429-56.
3. Prater, M.R., et al., *Role of Maternal Dietary Antioxidant Supplementation in Murine Placental and Fetal Limb Development.* Placenta, 2005.
4. Laudermilch, C.L., et al., *Placental improvement and reduced distal limb defects by maternal interferon-gamma injection in methylnitrosourea-exposed mice.* Birth Defects Res A Clin Mol Teratol, 2005. 73(9): p. 597-604.
5. Saito, S., *Cytokine network at the feto-maternal interface.* J Reprod Immunol, 2000. 47(2): p. 87-103.
6. Daher, S., et al., *Associations between cytokine gene polymorphisms and recurrent pregnancy loss.* J Reprod Immunol, 2003. 58(1): p. 69-77.
7. Wang, Y. and S.W. Walsh, *TNF alpha concentrations and mRNA expression are increased in preeclamptic placentas.* J Reprod Immunol, 1996. 32(2): p. 157-69.
8. Banerjee, S., et al., *Placental expression of IFN-{gamma} and its receptor IFN-{gamma}R2 fail to switch from early hypoxic to late normotensive development in pre-eclampsia.* J Clin Endocrinol Metab, 2004.
9. Delassus, S., et al., *Differential cytokine expression in maternal blood and placenta during murine gestation.* J Immunol, 1994. 152(5): p. 2411-20.
10. Dong, M., et al., *Placental imbalance of Th1- and Th2-type cytokines in preeclampsia.* Acta Obstet Gynecol Scand, 2005. 84(8): p. 788-93.
11. Goldman-Wohl, D.S., et al., *Lack of human leukocyte antigen-G expression in extravillous trophoblasts is associated with pre-eclampsia.* Mol Hum Reprod, 2000. 6(1): p. 88-95.
12. Hara, N., et al., *Altered expression of human leukocyte antigen G (HLA-G) on extravillous trophoblasts in preeclampsia: immunohistological demonstration with anti-HLA-G specific antibody "87G" and anti-cytokeratin antibody "CAM5.2".* Am J Reprod Immunol, 1996. 36(6): p. 349-58.
13. Le Bouteiller, P., F. Legrand-Abravanel, and C. Solier, *Soluble HLA-G1 at the materno-foetal interface--a review.* Placenta, 2003. 24 Suppl A: p. S10-5.
14. Abrahams, V.M., et al., *First trimester trophoblast cells secrete Fas ligand which induces immune cell apoptosis.* Mol Hum Reprod, 2004. 10(1): p. 55-63.
15. Marzi, M., et al., *Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy.* Clin Exp Immunol, 1996. 106(1): p. 127-33.
16. Hayakawa, S., et al., *Murine fetal resorption and experimental pre-eclampsia are induced by both excessive Th1 and Th2 activation.* J Reprod Immunol, 2000. 47(2): p. 121-38.
17. Daynes, R.A. and B.A. Araneo, *Contrasting effects of glucocorticoids on the capacity of T cells to produce the growth factors interleukin 2 and interleukin 4.* Eur J Immunol, 1989. 19(12): p. 2319-25.

18. Saito, S., et al., *Distribution of Th1, Th2, and Th0 and the Th1/Th2 cell ratios in human peripheral and endometrial T cells.* Am J Reprod Immunol, 1999. 42(4): p. 240-5.
19. Piccinni, M.P., et al., *Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions.* Nat Med, 1998. 4(9): p. 1020-4.
20. Hotchkiss, J.H., *Preformed N-nitroso compounds in foods and beverages.* Cancer Surv, 1989. 8(2): p. 295-321.
21. Hotchkiss, J.H., *A review of current literature on N-nitroso compounds in foods.* Adv Food Res, 1987. 31: p. 53-115.
22. Drablos, F., et al., *Alkylation damage in DNA and RNA--repair mechanisms and medical significance.* DNA Repair (Amst), 2004. 3(11): p. 1389-407.
23. Bochert, G., et al., *Embryotoxicity induced by alkylating agents: left-sided preponderance of paw malformations induced by acetoxymethyl-methylnitrosamine in mice.* Arch Toxicol, 1985. 56(3): p. 139-50.
24. Jacoby, R.F., et al., *K-ras oncogene mutations in rat colon tumors induced by N-methyl-N-nitrosourea.* Carcinogenesis, 1992. 13(1): p. 45-9.
25. Hall, A., *A biochemical function for ras--at last.* Science, 1994. 264(5164): p. 1413-4.
26. Glassner, B.J., et al., *DNA repair methyltransferase (Mgmt) knockout mice are sensitive to the lethal effects of chemotherapeutic alkylating agents.* Mutagenesis, 1999. 14(3): p. 339-47.
27. Sukumar, S. and M. Barbacid, *Specific patterns of oncogene activation in transplacentally induced tumors.* Proc Natl Acad Sci U S A, 1990. 87(2): p. 718-22.
28. Hsieh, L.L. and T.T. Hsieh, *Detection of aflatoxin B1-DNA adducts in human placenta and cord blood.* Cancer Res, 1993. 53(6): p. 1278-80.
29. Nagao, T., et al., *Induction of fetal malformations after treatment of mouse embryos with methylnitrosourea at the preimplantation stages.* Teratog Carcinog Mutagen, 1991. 11(1): p. 1-10.
30. Loch-Caruso, R. and C.S. Baxter, *DNA damage and repair in embryonic mouse limbs exposed to teratogenic doses of methylnitrosourea.* Mutat Res, 1984. 131(3-4): p. 147-55.
31. Tarr, M.J., et al., *The effects of methylnitrosourea on the immune system and hematopoietic system of adult specific pathogen free cats.* Chem Biol Interact, 1979. 28(2-3): p. 181-99.
32. Iannaccone, P.M., T.Y. Tsao, and L. Stols, *Effects on mouse blastocysts of in vitro exposure to methylnitrosourea and 3-methylcholanthrene.* Cancer Res, 1982. 42(3): p. 864-8.
33. Bossert, N.L. and P.M. Iannaccone, *Midgestational abnormalities associated with in vitro preimplantation N-methyl-N-nitrosourea exposure with subsequent transfer to surrogate mothers.* Proc Natl Acad Sci U S A, 1985. 82(24): p. 8757-61.

34. Platzek, T., et al., *Embryotoxicity induced by alkylating agents: 5. Dose-response relationships of teratogenic effects of methylnitrosourea in mice.* Arch Toxicol, 1988. 62(6): p. 411-23.
35. Prater, M.R., et al., *Reduced birth defects caused by maternal immune stimulation in methylnitrosourea-exposed mice: Association with placental improvement.* Birth Defects Res Part A Clin Mol Teratol, 2004. 70(11): p. 862-9.
36. Huang, P.H. and A. Catalano, *N-alkyl-N-nitrosourea induced secondary structural changes in DNA from rat embryos and fetal brains in vivo.* Teratog Carcinog Mutagen, 1992. 12(3): p. 135-53.
37. Akaike, M., et al., *Comparison of four spatial maze learning tests with methylnitrosourea-induced microcephaly rats.* Teratology, 1994. 49(2): p. 83-9.
38. Smith, S.B., C.B. Cooke, and K.L. Yielding, *Effects of the antioxidant butylated hydroxytoluene (BHT) on retinal degeneration induced transplacentally by a single low dosage of N-methyl-N-nitrosourea (MNU).* Teratog Carcinog Mutagen, 1988. 8(4): p. 175-89.
39. Nambu, H., et al., *Time-specific action of N-methyl-N-nitrosourea in the occurrence of retinal dysplasia and retinal degeneration in neonatal mice.* Pathol Int, 1998. 48(3): p. 199-205.
40. Punareewattana, K., et al., *Reduced birth defects caused by maternal immune stimulation may involve increased expression of growth promoting genes and cytokine GM-CSF in the spleen of diabetic ICR mice.* Int Immunopharmacol, 2003. 3(12): p. 1639-55.
41. Sharova, L.V., et al., *Maternal immune stimulation reduces both placental morphologic damage and down-regulated placental growth-factor and cell cycle gene expression caused by urethane: are these events related to reduced teratogenesis?* Int Immunopharmacol, 2003. 3(7): p. 945-55.
42. Chaouat, G., et al., *Control of fetal survival in CBA x DBA/2 mice by lymphokine therapy.* J Reprod Fertil, 1990. 89(2): p. 447-58.
43. Ashkar, A.A. and B.A. Croy, *Interferon-gamma contributes to the normalcy of murine pregnancy.* Biol Reprod, 1999. 61(2): p. 493-502.
44. Ashkar, A.A., J.P. Di Santo, and B.A. Croy, *Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy.* J Exp Med, 2000. 192(2): p. 259-70.
45. Croy, B.A., et al., *Histological studies of gene-ablated mice support important functional roles for natural killer cells in the uterus during pregnancy.* J Reprod Immunol, 1997. 35(2): p. 111-33.
46. Monk, J.M., et al., *Induction of murine spiral artery modification by recombinant human interferon-gamma.* Placenta, 2005. 26(10): p. 835-8.
47. Heikkinen, J., et al., *Phenotypic characterization of regulatory T cells in the human decidua.* Clin Exp Immunol, 2004. 136(2): p. 373-8.
48. Fallarino, F., et al., *Functional expression of indoleamine 2,3-dioxygenase by murine CD8 alpha(+) dendritic cells.* Int Immunol, 2002. 14(1): p. 65-8.

49. Munn, D.H., et al., *Prevention of allogeneic fetal rejection by tryptophan catabolism.* Science, 1998. 281(5380): p. 1191-3.
50. Boehm, U., et al., *Cellular responses to interferon-gamma.* Annu Rev Immunol, 1997. 15: p. 749-95.
51. Dalton, D.K., et al., *Multiple defects of immune cell function in mice with disrupted interferon-gamma genes.* Science, 1993. 259(5102): p. 1739-42.
52. Flynn, J.L., et al., *An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection.* J Exp Med, 1993. 178(6): p. 2249-54.
53. Haddad, E.K., et al., *Role of interferon-gamma in the priming of decidual macrophages for nitric oxide production and early pregnancy loss.* Cell Immunol, 1997. 181(1): p. 68-75.
54. Schaffer, M., et al., *Regulation of nitric oxide synthesis in wounds by IFN-gamma depends on TNF-alpha.* J Invest Surg, 2006. 19(6): p. 371-9.
55. Toapanta, F.R. and T.M. Ross, *Mouse strain-dependent differences in enhancement of immune responses by C3d.* Vaccine, 2004. 22(13-14): p. 1773-81.
56. Spearow, J.L., et al., *Genetic variation in physiological sensitivity to estrogen in mice.* APMIS, 2001. 109(5): p. 356-64.
57. Spearow, J.L., et al., *Genetic variation in susceptibility to endocrine disruption by estrogen in mice.* Science, 1999. 285(5431): p. 1259-61.
58. Holladay, S.D., et al., *Maternal immune stimulation in mice decreases fetal malformations caused by teratogens.* Int Immunopharmacol, 2002. 2(2-3): p. 325-32.
59. Sharova, L., et al., *Nonspecific stimulation of the maternal immune system. II. Effects on gene expression in the fetus.* Teratology, 2000. 62(6): p. 420-8.
60. Hrubec Tc, P.K.P.M.R.H.S.D., *Teratogen induced birth defects in mice: Role of maternal immune stimulation.* Current Topics in Toxicology, 2005.
61. Huang, S., et al., *Immune response in mice that lack the interferon-gamma receptor.* Science, 1993. 259(5102): p. 1742-5.
62. Nomura, T., S. Hata, and T. Kusafuka, *Suppression of developmental anomalies by maternal macrophages in mice.* J Exp Med, 1990. 172(5): p. 1325-30.
63. Grohmann, U., et al., *CTLA-4-Ig regulates tryptophan catabolism in vivo.* Nat Immunol, 2002. 3(11): p. 1097-101.
64. Punareewattana K, S.L.V.W.D.L.H.S.D., *Reduced birth defects caused by maternal immune stimulation in diabetic mice: Lack of correlation with placental gene expression.* Immunological Investigations, 2005. 34: p. 69-87.
65. Punareewattana, K., et al., *Reduced birth defects caused by maternal immune stimulation in diabetic ICR mice: lack of correlation with placental gene expression.* Immunol Invest, 2005. 34(1): p. 71-89.
66. Punareewattana, K. and S.D. Holladay, *Immunostimulation by complete Freund's adjuvant, granulocyte macrophage colony-stimulating factor, or interferon-gamma reduces severity of diabetic embryopathy in ICR mice.* Birth Defects Res Part A Clin Mol Teratol, 2004. 70(1): p. 20-7.
67. Holers, V.M., et al., *Complement C3 activation is required for antiphospholipid antibody-induced fetal loss.* J Exp Med, 2002. 195(2): p. 211-20.

68. Broome, C.S., et al., *An increase in selenium intake improves immune function and poliovirus handling in adults with marginal selenium status.* Am J Clin Nutr, 2004. 80(1): p. 154-62.
69. Loke, Y.W. and A. King, *Immunology of human placental implantation: clinical implications of our current understanding.* Mol Med Today, 1997. 3(4): p. 153-9.
70. Sharkey, A., *Cytokines and implantation.* Rev Reprod, 1998. 3(1): p. 52-61.
71. Hunt, J.S., M.G. Petroff, and T.G. Burnett, *Uterine leukocytes: key players in pregnancy.* Semin Cell Dev Biol, 2000. 11(2): p. 127-37.
72. Savion, S., et al., *A possible role for granulocyte macrophage-colony stimulating factor in modulating teratogen-induced effects.* Teratog Carcinog Mutagen, 1999. 19(3): p. 171-82.
73. Goldberg, M., L.S. Belkowski, and B.R. Bloom, *Regulation of macrophage function by interferon-gamma. Somatic cell genetic approaches in murine macrophage cell lines to mechanisms of growth inhibition, the oxidative burst, and expression of the chronic granulomatous disease gene.* J Clin Invest, 1990. 85(2): p. 563-9.
74. Holladay, S.D., et al., *Nonspecific stimulation of the maternal immune system. I. Effects On teratogen-induced fetal malformations.* Teratology, 2000. 62(6): p. 413-9.
75. Sharova, L.V., et al., *Immune stimulation in urethane-exposed pregnant mice increases expression level of spleen leukocyte genes for TGFbeta3 GM-CSF and other cytokines that may play a role in reduced chemical-induced birth defects.* Int Immunopharmacol, 2002. 2(10): p. 1477-89.
76. Salas-Vidal, E., C. Valencia, and L. Covarrubias, *Differential tissue growth and patterns of cell death in mouse limb autopod morphogenesis.* Dev Dyn, 2001. 220(4): p. 295-306.
77. Gorivodsky, M., et al., *Colony-stimulating factor-1 (CSF-1) expression in the uteroplacental unit of mice with spontaneous and induced pregnancy loss.* Clin Exp Immunol, 1999. 117(3): p. 540-9.
78. Holladay, S.D. and B.L. Blaylock, *The mouse as a model for developmental immunotoxicology.* Hum Exp Toxicol, 2002. 21(9-10): p. 525-31.
79. Renee Prater, M., et al., *Prebreeding maternal immunostimulation with Freund's complete adjuvant reduces placental damage and distal limb defects caused by methylnitrosourea.* Am J Reprod Immunol, 2006. 55(2): p. 145-55.
80. Fitzgerald, J.S., et al., *Signal transduction in trophoblast invasion.* Chem Immunol Allergy, 2005. 88: p. 181-99.
81. Gentleman, R.C., et al., *Bioconductor: open software development for computational biology and bioinformatics.* Genome Biol, 2004. 5(10): p. R80.
82. Bolstad, B.M., et al., *A comparison of normalization methods for high density oligonucleotide array data based on variance and bias.* Bioinformatics, 2003. 19(2): p. 185-93.
83. Huber, W., et al., *Parameter estimation for the calibration and variance stabilization of microarray data.* Stat Appl Genet Mol Biol, 2003. 2(1): p. Article3.

84. Huber, W., et al., *Variance stabilization applied to microarray data calibration and to the quantification of differential expression.* Bioinformatics, 2002. 18 Suppl 1: p. S96-104.
85. Smyth, G.K., *Linear models and empirical bayes methods for assessing differential expression in microarray experiments.* Stat Appl Genet Mol Biol, 2004. 3(1): p. Article3.
86. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. 25(4): p. 402-8.
87. Duncan, S.A., et al., *STAT signaling is active during early mammalian development.* Dev Dyn, 1997. 208(2): p. 190-8.
88. Takeda, K., et al., *Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality.* Proc Natl Acad Sci U S A, 1997. 94(8): p. 3801-4.
89. Furugori, K., et al., *Levels of hepatocyte growth factor and its messenger ribonucleic acid in uncomplicated pregnancies and those complicated by preeclampsia.* J Clin Endocrinol Metab, 1997. 82(8): p. 2726-30.
90. Bladt, F., et al., *Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud.* Nature, 1995. 376(6543): p. 768-71.
91. Nakamura, Y., et al., *Hepatocyte growth factor is a novel member of the endothelium-specific growth factors: additive stimulatory effect of hepatocyte growth factor with basic fibroblast growth factor but not with vascular endothelial growth factor.* J Hypertens, 1996. 14(9): p. 1067-72.
92. Nasu, K., et al., *Upregulation of human cytotrophoblast invasion by hepatocyte growth factor.* J Reprod Fertil Suppl, 2000. 55: p. 73-80.
93. Cross, J.C., D.G. Simmons, and E.D. Watson, *Chorioallantoic morphogenesis and formation of the placental villous tree.* Ann N Y Acad Sci, 2003. 995: p. 84-93.
94. Dash, P.R., et al., *Trophoblast apoptosis is inhibited by hepatocyte growth factor through the Akt and beta-catenin mediated up-regulation of inducible nitric oxide synthase.* Cell Signal, 2005. 17(5): p. 571-80.
95. Birchmeier, C., et al., *Met, metastasis, motility and more.* Nat Rev Mol Cell Biol, 2003. 4(12): p. 915-25.
96. Baykal, C., et al., *Expression of hepatocyte growth factor/scatter factor receptor in IUGR fetuses' placentas: an immunohistochemical analysis.* Fetal Diagn Ther, 2005. 20(4): p. 249-53.
97. Lala, P.K. and C. Chakraborty, *Factors regulating trophoblast migration and invasiveness: possible derangements contributing to pre-eclampsia and fetal injury.* Placenta, 2003. 24(6): p. 575-87.
98. Uehara, Y., et al., *Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor.* Nature, 1995. 373(6516): p. 702-5.
99. Huleihel, M., et al., *IL-1 activity is expressed differently during pregnancy in the rat uterine artery than in aortic or uterine tissues.* Am J Reprod Immunol, 2002. 48(3): p. 163-9.

100. Paulesu, L., R. Romagnoli, and E. Bigliardi, *Materno-fetal immunotolerance: is Interleukin-1 a fundamental mediator in placental viviparity?* Dev Comp Immunol, 2005. 29(5): p. 409-15.
101. Wang, X., et al., *Disruption of interleukin-18, but not interleukin-1, increases vulnerability to preterm delivery and fetal mortality after intrauterine inflammation.* Am J Pathol, 2006. 169(3): p. 967-76.
102. Voronov, E., et al., *IL-1 beta-deficient mice are resistant to induction of experimental SLE.* Eur Cytokine Netw, 2006. 17(2): p. 109-16.
103. Baker, J., et al., *Role of insulin-like growth factors in embryonic and postnatal growth.* Cell, 1993. 75(1): p. 73-82.
104. Gardner, R.L., et al., *Insulin-like growth factor-2 regulation of conceptus composition: effects of the trophectoderm and inner cell mass genotypes in the mouse.* Biol Reprod, 1999. 60(1): p. 190-5.
105. Constancia, M., et al., *Placental-specific IGF-II is a major modulator of placental and fetal growth.* Nature, 2002. 417(6892): p. 945-8.
106. DeChiara, T.M., A. Efstratiadis, and E.J. Robertson, *A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting.* Nature, 1990. 345(6270): p. 78-80.
107. Wang, Z.Q., et al., *Regulation of embryonic growth and lysosomal targeting by the imprinted Igf2/Mpr gene.* Nature, 1994. 372(6505): p. 464-7.
108. Lau, M.M., et al., *Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality.* Genes Dev, 1994. 8(24): p. 2953-63.
109. Wilkinson, D.G., S. Bhatt, and A.P. McMahon, *Expression pattern of the FGF-related proto-oncogene int-2 suggests multiple roles in fetal development.* Development, 1989. 105(1): p. 131-6.
110. Jakobovits, A., et al., *Two proto-oncogenes implicated in mammary carcinogenesis, int-1 and int-2, are independently regulated during mouse development.* Proc Natl Acad Sci U S A, 1986. 83(20): p. 7806-10.
111. Stamp, G., et al., *Nonuniform expression of a mouse mammary tumor virus-driven int-2/Fgf-3 transgene in pregnancy-responsive breast tumors.* Cell Growth Differ, 1992. 3(12): p. 929-38.
112. Rappolee, D.A., et al., *Expression and function of FGF-4 in peri-implantation development in mouse embryos.* Development, 1994. 120(8): p. 2259-69.
113. Zhong, W., et al., *FGF ligand family mRNA expression profile for mouse preimplantation embryos, early gestation human placenta, and mouse trophoblast stem cells.* Mol Reprod Dev, 2006. 73(5): p. 540-50.
114. Mansour, S.L., *Targeted disruption of int-2 (fgf-3) causes developmental defects in the tail and inner ear.* Mol Reprod Dev, 1994. 39(1): p. 62-7; discussion 67-8.
115. Friesel, R.E. and T. Maciag, *Molecular mechanisms of angiogenesis: fibroblast growth factor signal transduction.* Faseb J, 1995. 9(10): p. 919-25.
116. Green, P.J., F.S. Walsh, and P. Doherty, *Promiscuity of fibroblast growth factor receptors.* Bioessays, 1996. 18(8): p. 639-46.

117. Johnson, D.E. and L.T. Williams, *Structural and functional diversity in the FGF receptor multigene family.* Adv Cancer Res, 1993. 60: p. 1-41.
118. Vadivelu, S.K., et al., *Muscle regeneration and myogenic differentiation defects in mice lacking TIS7.* Mol Cell Biol, 2004. 24(8): p. 3514-25.
119. Arenander, A.T., et al., *TIS gene expression in cultured rat astrocytes: multiple pathways of induction by mitogens.* J Neurosci Res, 1989. 23(3): p. 257-65.
120. Arenander, A.T., et al., *TIS gene expression in cultured rat astrocytes: induction by mitogens and stellation agents.* J Neurosci Res, 1989. 23(3): p. 247-56.
121. Varnum, B.C., R.W. Lim, and H.R. Herschman, *Characterization of TIS7, a gene induced in Swiss 3T3 cells by the tumor promoter tetradecanoyl phorbol acetate.* Oncogene, 1989. 4(10): p. 1263-5.
122. Varnum, B.C., et al., *Nucleotide sequence of a cDNA encoding TIS11, a message induced in Swiss 3T3 cells by the tumor promoter tetradecanoyl phorbol acetate.* Oncogene, 1989. 4(1): p. 119-20.
123. Vietor, I., et al., *Perturbation of the tight junction permeability barrier by occludin loop peptides activates beta-catenin/TCF/LEF-mediated transcription.* EMBO Rep, 2001. 2(4): p. 306-12.
124. Vietor, I., et al., *TIS7 interacts with the mammalian SIN3 histone deacetylase complex in epithelial cells.* EMBO J, 2002. 21(17): p. 4621-31.
125. Scamuffa, N., et al., *Proprotein convertases: lessons from knockouts.* Faseb J, 2006. 20(12): p. 1954-63.
126. Recio, J.A. and G. Merlino, *Hepatocyte growth factor/scatter factor induces feedback up-regulation of CD44v6 in melanoma cells through Egr-1.* Cancer Res, 2003. 63(7): p. 1576-82.
127. Watanabe, K., et al., *A new crystal form of the NK1 splice variant of HGF/SF demonstrates extensive hinge movement and suggests that the NK1 dimer originates by domain swapping.* J Mol Biol, 2002. 319(2): p. 283-8.
128. Mbikay, M., et al., *Structure of the gene for the testis-specific proprotein convertase 4 and of its alternate messenger RNA isoforms.* Genomics, 1994. 20(2): p. 231-7.
129. Hasegawa, Y., et al., *A RecA-mediated exon profiling method.* Nucleic Acids Res, 2006. 34(13): p. e97.
130. Miki, T., et al., *Determination of ligand-binding specificity by alternative splicing: two distinct growth factor receptors encoded by a single gene.* Proc Natl Acad Sci U S A, 1992. 89(1): p. 246-50.
131. Uehara, Y., et al., *Rescue of embryonic lethality in hepatocyte growth factor/scatter factor knockout mice.* Genesis, 2000. 27(3): p. 99-103.
132. Muntener, M. and Y.C. Hsu, *Development of trophoblast and placenta of the mouse. A reinvestigation with regard to the in vitro culture of mouse trophoblast and placenta.* Acta Anat (Basel), 1977. 98(3): p. 241-52.

APPENDIX A

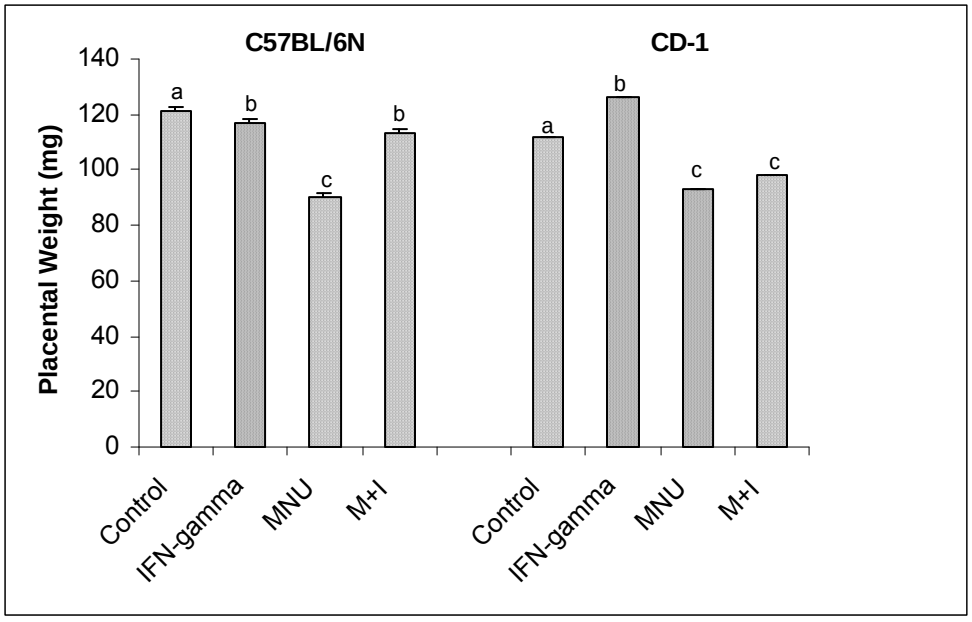


Figure 1: Comparison of Placental Weight in C57BL/6N and CD-1 Mice
Placental weight (mg ± SEM) means per litter from C57BL/6N and CD-1 dams in four experimental groups: Control (C57BL/6N n=14/CD-1 n=11), IFN-γ (n=7/n=13), MNU (n=18/16), and MNU+IFN-γ (n=9/n=8). MNU (20 mg/kg) was given by IP injection on GD9. IFN-γ (1.0 x 103 IU) was administered IP in a single dose on GD 7. All dams were sacrificed on GD14. Within mouse strains, bars with different letters represent a statistically significant difference.

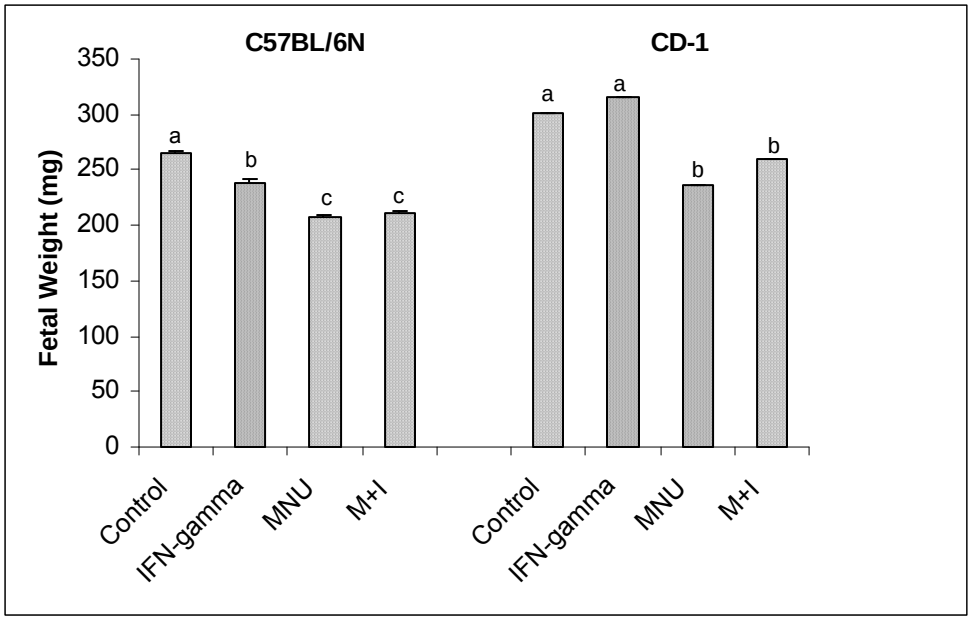


Figure 2: Comparison of Fetal Weight in C57BL/6N and CD-1 Mice
Fetal weight (mg ± SEM) from C57BL/6N and CD-1 dams that were exposed to four different treatment groups: Control (C57BL/6N n=14/CD-1 n=11), IFN-γ (n=7/n=13), MNU (n=18/16), and MNU+IFN-γ (n=9/n=8). MNU (20 mg/kg) was given by IP injection on gestation day 9 (GD9). IFN-γ (1.0 x 103 IU) was administered IP in a single dose on GD 7. All dams were sacrificed on GD14. Within mouse strains, bars with different letters represent a statistically significant difference.

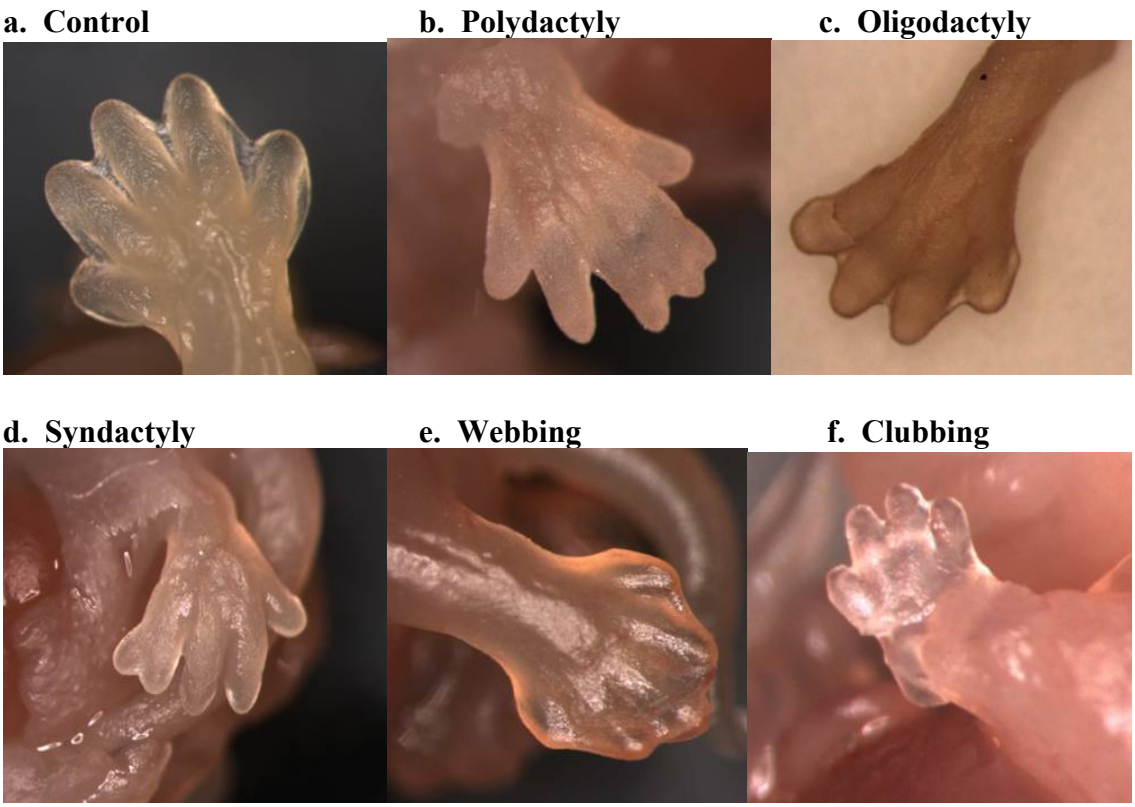


Figure 3: Distal Limb Defects Observed in MNU-Exposed Mice
Distal limb defects seen in MNU-exposed C57BL/6N mice as compared to control (a). b: Polydactyly was defined as more than five digits per distal limb. c: Oligodactyly was defined as fewer than five digits per distal limb (also seen in f). d: Syndactyly was defined as the fusion of two or more digits (also seen in b). e: Webbing was defined as a tissue membrane connecting the digits at least half way to the distal tips. f: Clubbing was defined by a bulbous palm with microdactyl digits.
These defects observed in C57BL/6N mice are representative of those produced in CD-1 experimental mice.

a. Control (200x)

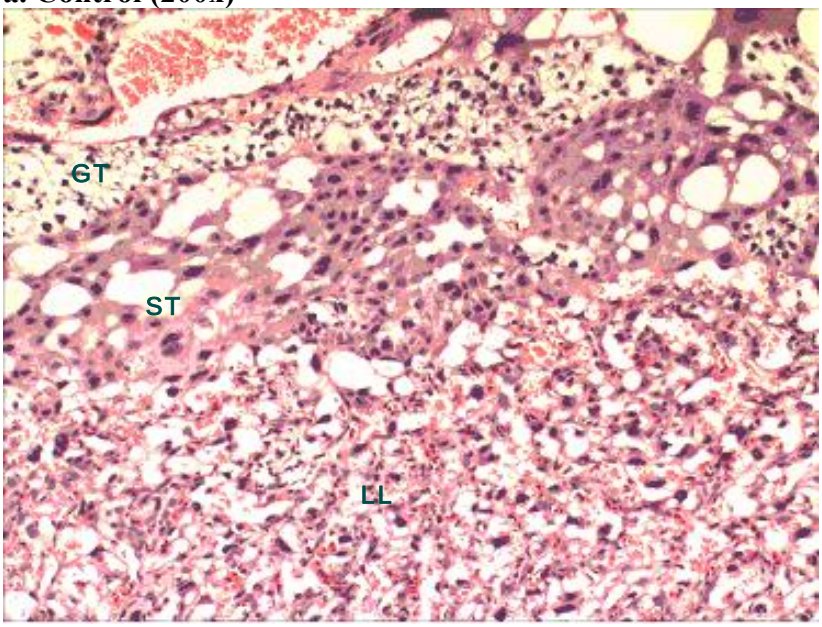
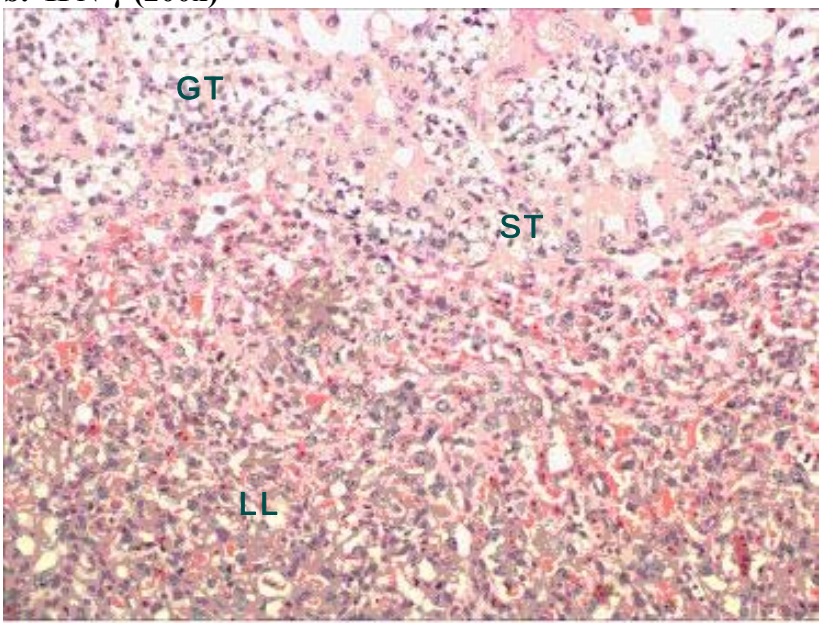


Figure 4: H&E sections of GD14 placenta from C57BL/6N Dams
Hematoxylin and eosin sections of gestation day 14 placenta from C57BL/6N dams in four different treatment groups: Control, IFN-γ, MNU, and MNU+IFN-γ. MNU (20 mg/kg) was given to dams through IP injection on gestation GD9. IFN-γ (1.0 x 103 IU) was administered IP in a single dose on GD 7. All dams were sacrificed on GD14. These sections are representative of the outcome seen in the same treatment groups in CD-1 mice. Labels within the sections are defined as follows, LL: Labryrinthine layer; GT: Glycogen trophoblast layer; ST: Syncitiotrophoblast layer.
a: Control placentas (200x) rarely displayed occasional necrotic or pyknotic trophoblasts and endothelial cells, typical in the tissue at this point in gestation.

b. IFN-γ (200x)



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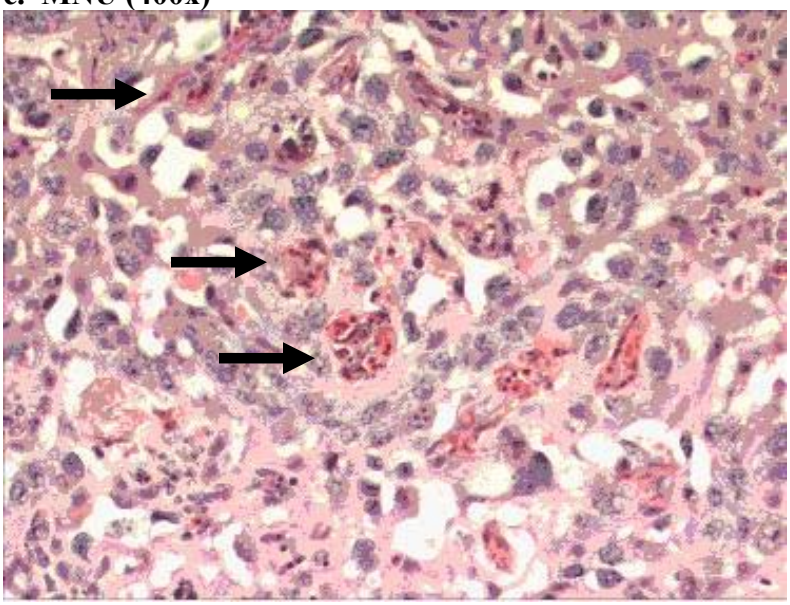
b: IFN-γ-treated placentas (200x) displayed occasional mild suppurative inflammatory infiltrates superficial to the trophoblast giant cell later. Rare evidence of necrotic cellular debris was observed in the labyrinthine and trophoblast layers.

c. MNU (400x)



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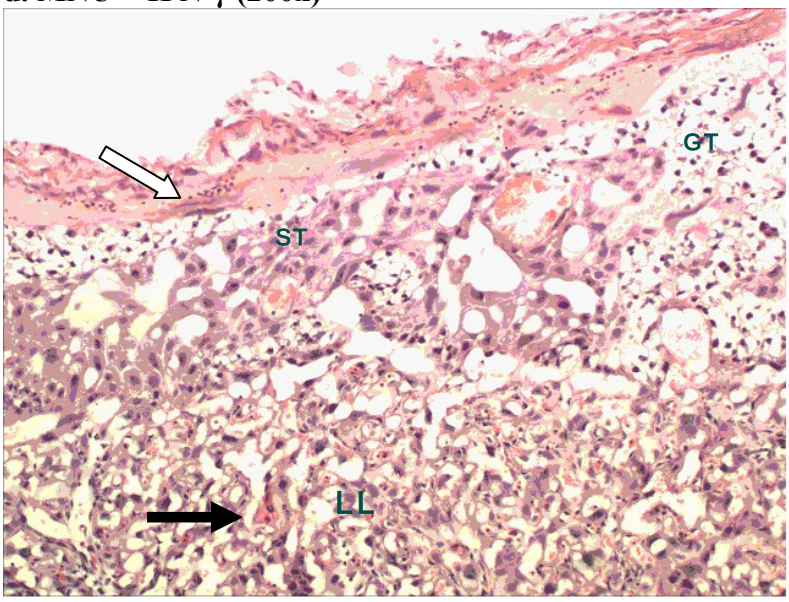
c: MNU-treated placentas (400x) showed multifocal areas of necrosis targeting the endothelium in the labryrinthine layer with karyolysis and pyknosis, cellular fragmentation, hypereosinophilia and collapse of vascular patency (see arrows in Fig. 4c). Necrotic trophoblasts were also scattered throughout the syncitial layer and in the giant cell layer with mild suppurative inflammation and dystrophic mineralization.

d. MNU + IFN-γ (200x)



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d: Placentas from MNU+IFN-γ-treated dams (200x) displayed a hybrid of the aforementioned lesions along with neutrophilic infiltration of the trophoblast giant cell layer (see white arrow in Fig. 4d) and rare focal areas of necrosis in the labyrinthine layer (see black arrow in Fig. 4d).

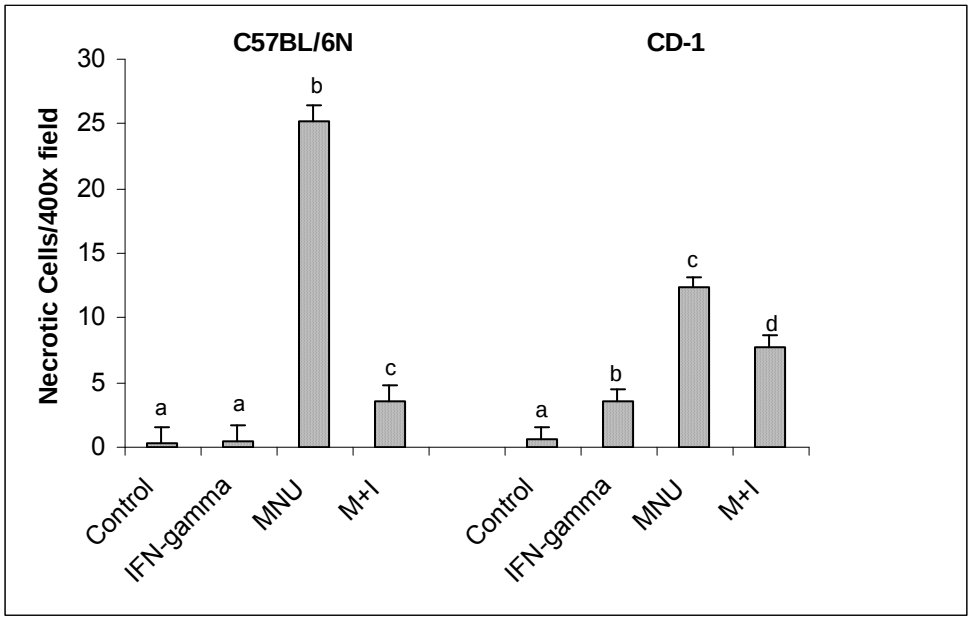

Figure 5: Endothelial Cell Loss in Labyrinthine Placenta
Numbers of dead endothelial cells per ten 400x fields of labyrinthine placenta in C57BL/6N and CD-1 mice following exposure to MNU and/or IFN-γ. MNU (20 mg/kg) was given by IP injection on GD9. IFN-γ (1.0 x 103 IU) was administered IP in a single dose on GD 7. All dams were sacrificed on GD14. Within mouse strains, bars with different letters represent a statistically significant difference.

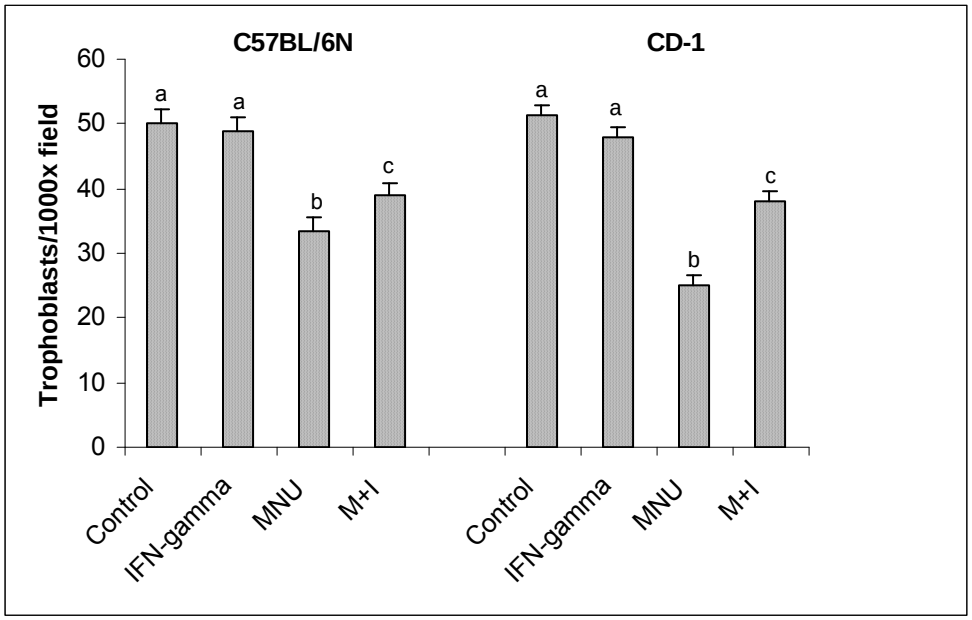

Figure 6: Trophoblast Viability in Syncitiotrohpoblast Placenta
Numbers of viable trophoblast cells per ten 1000x fields in the syncitiotrophoblast layer of placentas from C57BL/6N and CD-1 mice, following exposure to MNU and/or IFN-γ. MNU (20 mg/kg) was given by IP injection on GD9. IFN-γ (1.0 x 103 IU) was administered IP in a single dose on GD 7. All dams were sacrificed on GD14. Within mouse strains, bars with different letters represent a statistically significant difference.

Table 1. Absolute Body and Limb Length in C57BL/6N and in CD-1 Fetuses

	Treatment	Fetal Body Length (mm)	Limb measurements (mm)			
			Right Front	Left Front	Right Hind	Left Hind
C57BL/6N	Control	10.78 ± 0.15[a]	3.57 ± 0.07[a]	3.53 ± 0.08[a]	3.81 ± 0.07[a]	3.94 ± 0.07[a]
	IFN-γ	11.18 ± 0.15[a]	3.55 ± 0.07[a]	3.54 ± 0.08[a]	3.86 ± 0.07[a]	3.82 ± 0.07[a]
	MNU	10.30 ± 0.15[b]	2.99 ± 0.07[b]	3.09 ± 0.08[b]	3.38 ± 0.07[b]	3.43 ± 0.07[b]
	MNU+ IFN-γ	9.81 ± 0.15[c]	2.76 ± 0.07[b]	2.72 ± 0.08[c]	3.18 ± 0.07[b]	3.10 ± 0.07[c]
CD-1	Control	12.15 ± 0.15[a]	3.62 ± 0.07[a]	3.73 ± 0.07[a]	3.86 ± 0.07[a]	4.07 ± 0.07[a]
	IFN-γ	13.30 ± 0.15[a]	3.56 ± 0.07[a]	3.57 ± 0.07[a]	3.87 ± 0.07[a]	3.86 ± 0.07[a]
	MNU	11.63 ± 0.15[b]	3.18 ± 0.07[b]	3.31 ± 0.07[b]	3.34 ± 0.07[b]	3.62 ± 0.07[b]
	MNU+ IFN-γ	11.97 ± 0.15[b]	2.80 ± 0.07[c]	2.83 ± 0.07[c]	3.19 ± 0.07[b]	3.22 ± 0.07[c]

Numbers within a column of a group of mice that have a different superscript letter indicate statistical significance $p < 0.05$.

Table 2. Limb Length to Body Length Ratios in C57BL/6N and in CD-1 Fetuses

	Treatment	Ratio of Limb Length/Body Length			
		Right Front	Left Front	Right Hind	Left Hind
C57BL/6N	Control	0.331 ± 0.006[a]	0.327 ± 0.007[a]	0.353 ± 0.008[a]	0.366 ± 0.006[a]
	IFN-γ	0.318 ± 0.006[a]	0.318 ± 0.007[a]	0.346 ± 0.008[a]	0.343 ± 0.006[b]
	MNU	0.292 ± 0.006[b]	0.301 ± 0.007[b]	0.331 ± 0.008[a]	0.335 ± 0.006[b]
	MNU+ IFN-γ	0.282 ± 0.006[b]	0.278 ± 0.007[b]	0.326 ± 0.008[a]	0.316 ± 0.006[c]
CD-1	Control	0.298 ± 0.006[a]	0.307 ± 0.006[a]	0.318 ± 0.007[a]	0.335 ± 0.006[a]
	IFN-γ	0.269 ± 0.006[b]	0.269 ± 0.006[b]	0.292 ± 0.007[a]	0.291 ± 0.006[b]
	MNU	0.274 ± 0.006[b]	0.285 ± 0.006[a]	0.288 ± 0.007[a]	0.312 ± 0.006[a]
	MNU+ IFN-γ	0.232 ± 0.006[c]	0.237 ± 0.006[c]	0.268 ± 0.007[b]	0.270 ± 0.006[b]

Numbers within a column of a group of mice that have a different superscript letter indicate statistical significance $p < 0.05$.

Table 3. Morphological Malformations seen in C57 Mice at Gestation Day 14

Deformity	Syndactyly		Oligodactyly		Polydactyly		Webbing		Clubbing		Total F+H/ #embryos	# litters
Limb:	Fore	Hind	Fore	Hind	Fore	Hind	Fore	Hind	Fore	Hind		
Control	0	6	0	0	0	0	1	6	0	0	13/117	14
IFN-γ	5	1	0	1	0	0	0	12	0	1	20/51	7
MNU	15	16	17	29	3	2	2	31	2	4	121/94	18
MNU+IFNγ	2	6	17	18	0	0	0	6	3	2	54/45	9

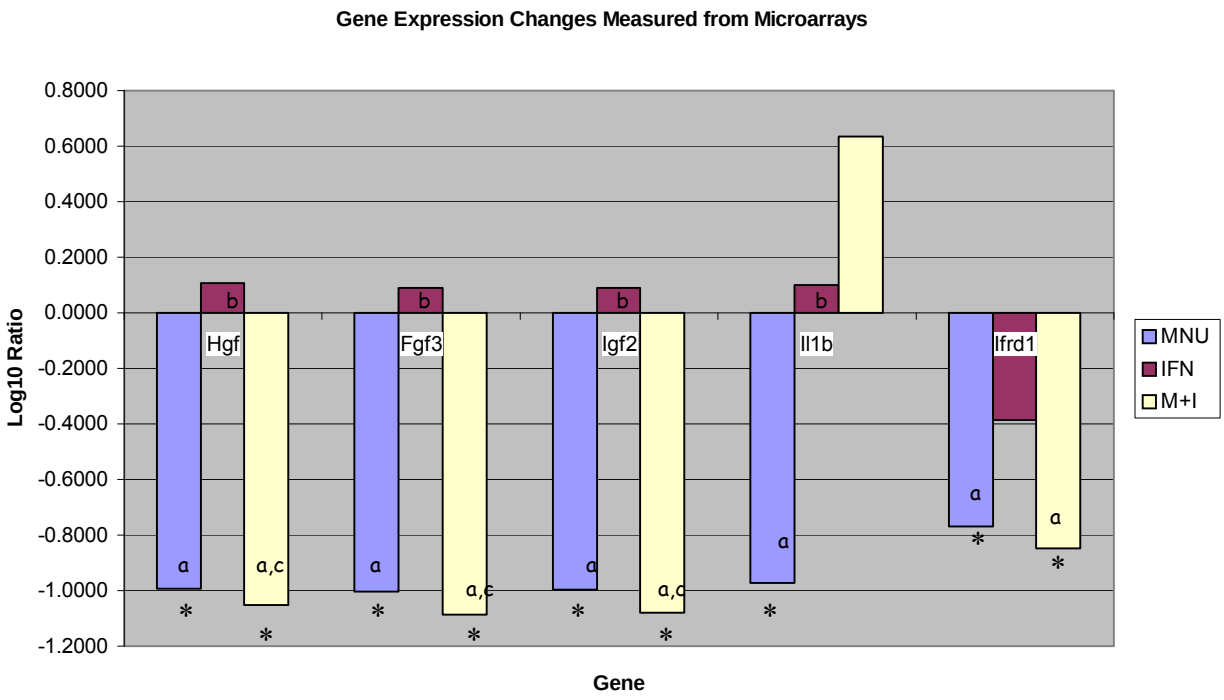

Figure 7. Gene Expression Changes Measured from Microarrays

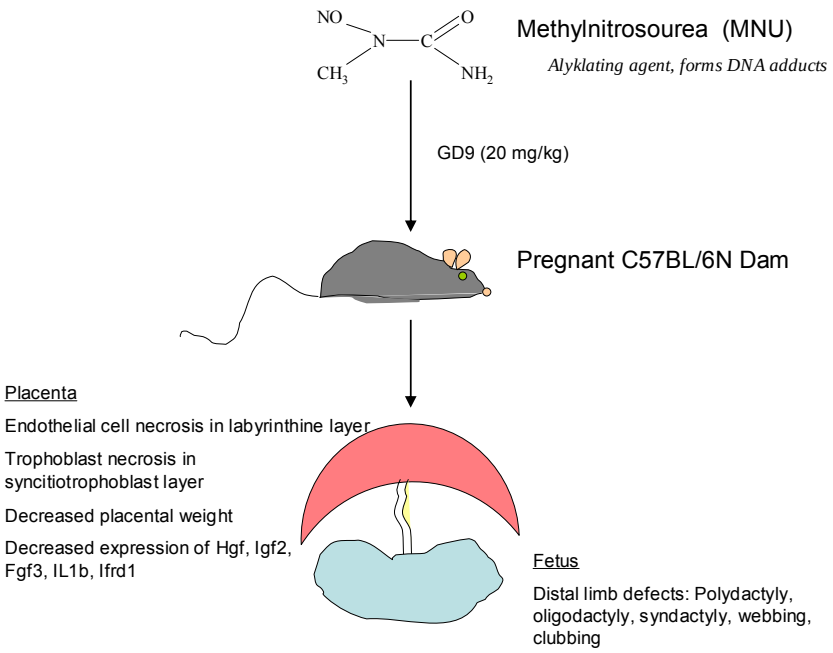

Figure 8. Proposed Mechanism of Observed Teratogenesis after MNU Administration on GD9

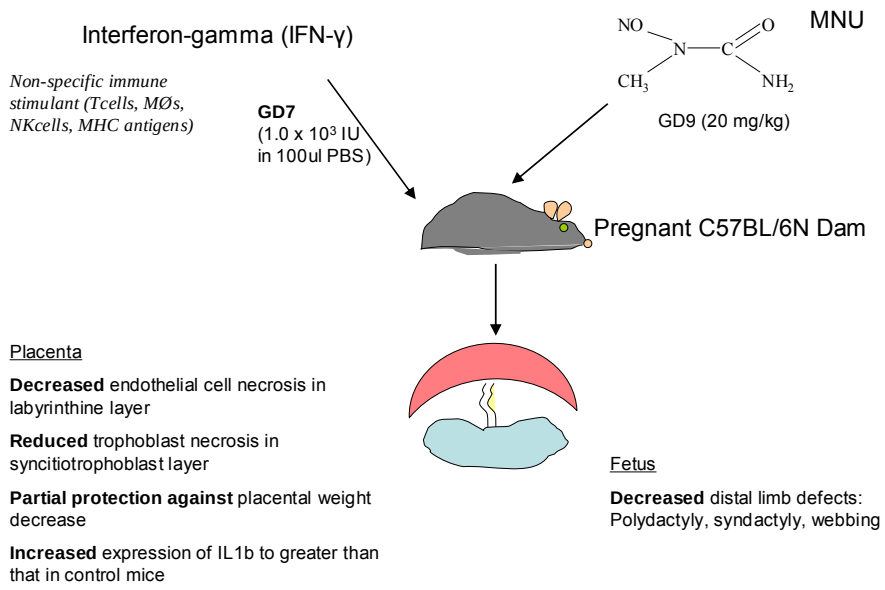

Figure 9. Proposed Mechanism of Birth Defect Amelioration due to Maternal Immune Stimulation with IFN-γ in MNU-treated Dams

VITA

Chelsea Lee Laudermilch was born to Thomas and Brenda Boyll in Peru, Indiana. She graduated from high school in 2001 and then attended Indiana Wesleyan University in Marion, Indiana and received her Bachelor of Science in biology in 2004. There, she met her husband to be, Ben. After their marriage in 2004, she followed him to the Virginia-Maryland Regional College of Veterinary Medicine, where he was a veterinary student. While working as a laboratory specialist in the Developmental Immunotoxicology lab of Dr. Renee Prater, she also pursued her M.S. in IFN-γ-mediated birth defect reduction. She and her husband are expecting their first baby in March, and will settle their family in northeastern Pennsylvania where her husband will join his father in practicing large animal veterinary medicine. Chelsea intends to one day teach biological sciences at a private Christian school or university.