Sex-Dependent Influence of Developmental Toxicant Exposure on Group B *Streptococcus*-Mediated Preterm Birth in a Murine Model

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Tianbing Ding, PhD¹, Lauren A. Lambert¹, David M. Aronoff, MD^{1,2},
Kevin G. Osteen, PhD^{1,3,4}, and Kaylon L. Bruner-Tran, PhD¹

Abstract

Infectious agents are a significant risk factor for preterm birth (PTB); however, the simple presence of bacteria is not sufficient to induce PTB in most women. Human and animal data suggest that environmental toxicant exposures may act in concert with other risk factors to promote PTB. Supporting this “second hit” hypothesis, we previously demonstrated exposure of fetal mice (F1 animals) to the environmental endocrine disruptor 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) leads to an increased risk of spontaneous and infection-mediated PTB in adult animals. Surprisingly, adult F1_{males} also confer an enhanced risk of PTB to their control partners. Herein, we used a recently established model of ascending group B *Streptococcus* (GBS) infection to explore the impact of a maternal versus paternal developmental TCDD exposure on infection-mediated PTB in adulthood. Group B *Streptococcus* is an important contributor to PTB in women and can have serious adverse effects on their infants. Our studies revealed that although gestation length was reduced in control mating pairs exposed to low-dose GBS, dams were able to clear the infection and bacterial transmission to pups was minimal. In contrast, exposure of pregnant F1_{females} to the same GBS inoculum resulted in 100% maternal and fetal mortality. Maternal health and gestation length were not impacted in control females mated to F1_{males} and exposed to GBS; however, neonatal survival was reduced compared to controls. Our data revealed a sex-dependent impact of parental TCDD exposure on placental expression of Toll-like receptor 2 and glycogen production, which may be responsible for the differential impact on fetal and maternal outcomes in response to GBS infection.

Keywords

TCDD, TLR2, pregnancy, placenta, glycogen

Introduction

An unintended consequence of industrialization has been the contamination of our environment with an astonishing number of potentially harmful compounds. More than 80 000 chemicals have been released into our environment since the Toxic Substances Control Act of 1976; however, only a limited number of these agents have undergone controlled experimental evaluation.¹ Among those that have been examined, many have been found to disrupt normal steroid action and are thus termed endocrine-disrupting chemicals (EDCs). Exposure to EDCs is ubiquitous, and humans have little ability to completely avoid such compounds; thus, understanding the health consequences of toxicant exposure is an important area of research. Although the adverse consequences of exposure of pregnant women to environmental pollution has been well documented,^{2,3} the long-term effects of such exposure on the surviving offspring’s adult reproductive health are limited. Nevertheless, exposures to EDCs during the neonatal period are recognized as being more damaging compared to adult exposures, often with lifelong and even

transgenerational consequences.^{4,5} Several studies in mice have revealed an enhanced susceptibility to infection following a developmental exposure to environmental EDCs. For example, Lawrence and colleagues demonstrated that influenza infection in adult mice was more severe in animals with a history of in utero 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure.⁶ Additional studies from this same group demonstrated

¹ Department of Obstetrics and Gynecology, Women’s Reproductive Health Research Center, Vanderbilt University Medical Center, Nashville, TN, USA

² Division of Infectious Disease, Department of Medicine, Vanderbilt University Medical Center, Nashville, TN, USA

³ Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN, USA

⁴ VA Tennessee Valley Healthcare System, Nashville, TN, USA

Corresponding Author:

Kaylon L. Bruner-Tran, Women’s Reproductive Health Research Center, Vanderbilt University School of Medicine, 1161 21st Ave S MCN B-1100, Nashville, TN 37232, USA.

Email: kaylon.bruner-tran@vanderbilt.edu

that, following early life exposure to TCDD, female mice exhibited a more profound exacerbation of autoimmune symptoms in adulthood compared to their male littermates.⁷ Indeed, numerous studies indicate a sex-dependent impact of EDC exposure on various adult outcomes.⁸⁻¹⁰ Thus, examination of the potential sex-dependent effects of developmental TCDD exposure on adult pregnancy response to infection is warranted.

To this end, we previously examined the sex-specific impact of developmental TCDD exposure on pregnancy outcomes in a subsequent adult pregnancy. Specifically, in our model, pregnant C57BL/6 mice are exposed to a single TCDD dose on embryonic day 15.5 (E15.5). Pregnancy in the exposed dam is not impacted, and pups (F1 mice) are born as expected on E20. However, nearly half of adult male and female F1 mice exhibit infertility when mated to control partners.¹¹⁻¹³ Furthermore, we have documented spontaneous preterm birth (PTB; parturition <E19) in up to 47% of fertile F1_{females}, coincident with reduced uterine progesterone sensitivity and a heightened inflammatory response during late pregnancy.¹²⁻¹⁴ Moreover, following an unexpected mouse parvovirus (MPV) outbreak in our colony, 100% of mice with a history of toxicant exposure delivered preterm, while control animals were unaffected.¹² These data suggest toxicant-exposed animals exhibit an overly robust response to MPV infection in adulthood, similar to the hyperinflammatory response to influenza previously described by the Lawrence laboratory.⁶

Group B *Streptococcus* (GBS; *S. agalactiae*) is a well-recognized pathogen associated with the risk of PTB in women.¹⁵ Rectal and/or vaginal colonization with GBS occurs in 25% to 50% of pregnant women^{16,17} and is a major risk factor for ascending infection of the gravid uterus and its consequences, including chorioamnionitis, premature rupture of the membranes, stillbirth, neonatal sepsis, and PTB.^{15,18} Although GBS colonization is the major risk factor for invasive perinatal infection,¹⁹ only a subset of colonized patients have infection or experience PTB.^{20,21} The basis for this clinical variability is unknown. In light of the murine data described earlier, it can be speculated that the risk of infection-mediated PTB may be significantly enhanced by the concomitant presence of other risk factors (eg, genetic or environmental factors). It is also possible that the preconception health of the father is capable of influencing susceptibility to infection in his pregnant partner as a consequence of placental dysfunction.¹³

As noted earlier, we have documented an enhanced risk of spontaneous PTB in the control partners of F1_{males}. This unexpected outcome was associated with an inflammatory preconception testicular environment¹¹ and premature placental inflammation during pregnancy.^{13,22} Seminal studies conducted in 1985 by Barton and colleagues demonstrated that fetal development is markedly stunted in artificially created blastocysts containing only paternally derived genetic material; however, these “embryos” exhibited extraembryonic tissues and placental development.^{23,24} Several additional studies have now confirmed the significant contribution of paternal genes to normal placental development.²⁴⁻²⁷ Herein, in order to visually

illustrate the significant paternal contribution to placental tissues, beyond that which occurs via the fetal genotype, we utilized male and female mice heterozygous for green fluorescent protein (GFP^{+/-}). These transgenic mice express GFP in all cells other than erythrocytes; thus, mating animals discordant for GFP is useful to delineate parent of origin within the placenta. This simple experiment allowed for the visualization of the contribution of the father to the placental junctional zone (JZ) and fetal membranes, supporting previous studies examining the role of imprinted genes on placental development.^{24,28,29} Since these tissues serve as the final barrier between the external environment and incubating fetus,^{30,31} it is likely that the maternal and paternal phenotype would each be capable of influencing the clinical course of an ascending GBS infection during pregnancy. Therefore, using a recently developed mouse model of ascending GBS infection,³² we assessed the influence of developmental TCDD exposure of either parent on the risk of PTB following maternal GBS infection. Our studies revealed sex-specific differences between the placental phenotypes of F1_{female} mice and control females mated to F1_{males}, which significantly impacted pregnancy and fetal outcomes following maternal GBS infection. Understanding host factors that play a role in response to infection is paramount to developing more effective strategies to identify patients who are truly at risk of adverse outcomes.

Methods

Assessment of Placentae From GFP^{+/-} Transgenic Mice

Transgenic mice, heterozygous for GFP (C57BL/6-Tg(CAG-EGFP)1Osb/J; herein, referred to as GFP^{+/-}) were originally purchased from Jackson Laboratories (Bar Harbor, Maine). For the current study, male and female GFP^{+/-} mice were obtained from our in-house colony and mated to wild-type animals. Pregnant mice were euthanized on E13.5 and implant sites removed in toto with placenta and fetus intact. Tissues were frozen in Optimal Cutting Temperature compound (OCT) and cryosections subjected to fluorescence microscopy. Fluorescence was measured using the open source Fiji software (ImageJ³³; as previously described by Fitzpatrick).³⁴ Multiple placentae were assessed for each group. Using the freeform selection tool of Fiji, areas encompassing the decidual zone (DZ), JZ, and labyrinth zone (LZ) were selected and individually assessed for fluorescence. The corrected total cell fluorescence (CTCF) was calculated by the following formula:

$$\text{CTCF} = \text{Integrated Density} - (\text{Area of selected cells} \times \text{Mean fluorescence of background}).$$

In Utero TCDD Exposure Model

C57bl/6 mice were originally obtained from Envigo (Indianapolis, Indiana). At 10 to 12 weeks of age, virgin C57bl/6 females were mated with breeder males of similar age.

Following identification of a vaginal plug (E0.5), males were removed and females monitored for pregnancy. Pregnant mice (F0) were exposed to TCDD (10 µg/kg) in corn oil or vehicle alone by gavage on E15.5 (when organogenesis is complete). TCDD was given at this time, and dose was not overtly teratogenic and gestation length was not affected in the F0 animals; pups (F1 mice) were born on E20 ± 12 hours. The relatively high dose selected for our studies reflects the reduced sensitivity of mice to TCDD compared to humans³⁵⁻³⁷ and the inefficient transfer of this toxicant across the mouse placenta.³⁷ All studies involving mice were approved by Vanderbilt University's Institutional Animal Care and Use Committee.

Monitoring Pregnancy in Experimental Animals

Control females mated to control or F1_{males} and F1_{females} mated to control males were examined each morning for a vaginal plug, at which time (E0.5) males were removed. Females exhibiting signs of pregnancy (weight gain, nipple prominence) were inoculated with GBS or vehicle on E15.5 (described subsequently) and monitored for parturition by remote surveillance until delivery.

Bacterial Strains and Culture Conditions

The capsular serotype V GBS strain GB37, obtained from a human case of neonatal sepsis,³⁸ was cultured on blood agar plates at 37°C in ambient air overnight. Bacteria were subcultured from blood agar plates into Todd-Hewitt broth (THB) and incubated (aerobically, shaking at 200 rpm) at 37°C in ambient air overnight. The next morning, bacterial density was measured spectrophotometrically at an optical density of 600 nm (OD₆₀₀), and bacterial numbers were determined with a coefficient of 1 OD₆₀₀ = 10⁹ colony-forming unit (CFU)/mL.

Mouse Model of Ascending GBS Infection

Our model of ascending GBS infection of pregnant mice was modeled after the studies described by Randis et al.³² and modified by our team.³⁹ Briefly, pregnant mice (E15.5) were anesthetized with isoflurane and 50 µL of inocula containing ≥10² CFUs in THB medium plus 10% gelatin was introduced into the vagina. Sham controls were inoculated with 50 µL of THB medium containing 10% gelatin. Animals were housed singly until spontaneous delivery or were euthanized on embryonic day 17.5 (E17.5). Necropsy was performed on euthanized mice and multiple tissues collected (blood, vagina, uterus, cervix, placenta, and fetus).

Immunohistochemistry/Histochemistry

Immunolocalization of GBS. Immunohistochemical localization of GBS was performed by Vanderbilt Translational Pathology Shared Resource (TPSR) core laboratory using the Leica Bond Max autostainer (Buffalo Grove, IL). The rabbit polyclonal anti-GBS antibody was obtained from Abcam (Cambridge, MA; Cat #ab53584; 1 mg/mL) and used at a dilution of 1:500 following ER2 (EDTA) antigen retrieval for 5 minutes.

Periodic acid–Schiff histochemistry. Periodic acid–Schiff (PAS) staining was also performed by Vanderbilt's TPSR using the Artisan PAS Stain Kit from DakoCytomation (Glostrup, Denmark; Cat#AR16511) as recommended by the manufacturer.

Toll-like receptor 2 immunolocalization. Immunohistochemical staining of Toll-like receptor 2 (TLR2) was performed in our laboratory by standard methodology for formalin-fixed, paraffin-embedded tissues. Briefly, tissues were deparaffinized with xylene and rehydrated in serial dilutions of ethanol. After heat-activated antigen retrieval, primary antibody was applied (Sigma #PRS3135 [1 mg/mL]; used at 1:500 in phosphate-buffered saline [PBS]/Tween) and slides incubated overnight at 4°C in a humid chamber. Primary antibody was removed, slides rinsed in PBS/Tween, and biotinylated antirabbit secondary antibody applied (Biogenex, Fremont, CA; Cat #HK336-9R; used as supplied). After a 1-hour incubation at room temperature, slides were washed in PBS and TLR2 visualized using the Dako Envision+ HRP/DAB System (DakoCytomation) followed by counterstaining with Mayer hematoxylin. After staining, all slides were dehydrated, cleared, and cover-slipped for morphological analysis. For the negative control, the primary antibody was omitted.

Morphometry

Histopathological assessments were performed using an Olympus (Tokyo, Japan) BX51 microscope system and images captured using an Olympus DP71 digital camera. Staining intensity was assessed using Fiji software (ImageJ; Rockville, MD)³³ as previously described by Nguyen et al.⁴⁰ Reciprocal intensity was calculated by the formula: $r = 255 - y$, where y is the mean intensity of each image and 255 is the maximum intensity value of an RGB image analyzed in ImageJ (Fiji).

Periodic acid–Schiff staining. The PAS stained slides were scanned at low power, and the area with the most intense staining was photographed at a magnification of ×100, encompassing all regions of the placenta. The same region on a sister section, subjected to PAS-D staining, was similarly photographed. Following assessment of staining intensity by ImageJ, the reciprocal staining intensity of PAS-D placenta was subtracted from the reciprocal intensity value obtained following PAS staining in the absence of diastase. The resulting value was used for statistical analysis for the presence of glycogen in each sample. A single image was used from each available placenta (N = 3-7) from 4 dams per group.

Group B Streptococcus staining. All slides were scanned at low power to identify areas with GBS localization. From each placenta, 2 nonoverlapping fields exhibiting the greatest GBS colonization were photographed at a magnification of ×400. All available placenta (N = 4-5) from 5 dams per group were utilized.

Figure 1. Fluorescence microscopy of placentae arising from GFP^{+/+} females or GFP^{+/+} males. The GFP^{+/+} females were mated to wild-type males (A-B, E-F), and GFP^{+/+} males were mated to wild-type females (C-D, G-H). Pregnant animals were euthanized on E13.5 and placenta, decidua, and feti removed in toto and frozen samples subjected to fluorescence microscopy (A-D). Corresponding H&E images are shown for each placenta (E-H). Original magnification, $\times 1.25$. The corrected total cell fluorescence, as determined by ImageJ analysis, is shown in (I). ** $P < .0001$, * $P = .05$ compared to (A). GFP indicates green fluorescence protein; H&E, hematoxylin and eosin.

Staining of TLR2. All available placentae ($N = 3-7$) from 5 dams per group were photographed at $\times 40$. From each placenta, using the freeform selection tool of Fiji, areas encompassing the DZ, JZ, and LZ were selected and individually assessed for TLR2 localization.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis of TLR2

Total RNA was isolated from all frozen placentae with attached decidua tissues using TRIzol (Invitrogen, Carlsbad, California) and purified using the RNeasy Mini Kit (Qiagen, Valencia, California). Complementary DNA from 1 μ g of total RNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) and random decamer primers. Reactions were performed in triplicate in a Bio-Rad CFX96 real-time thermocycler system. The ribosomal protein L13a (Rpl13a) gene was used as an endogenous control. Results were evaluated using the $\Delta\Delta C_t$ method, where delta was calculated as (Target C_t) – (Rpl13a C_t), and the relative quantity of target gene expression was calculated by the $\Delta\Delta C_t$ as $2^{-([\text{experiment sample delta } C_t] - [\text{control sample delta } C_t])}$.

Primers

Tlr2: Forward: GGTTGTTCCCTGTGTTGC; reverse: AAAGTGGTTGTCGCCTGCT(predicted size, 120 base pair [bp]).

Rpl13a: Forward: TACCAGAAAGTTTGCTTACCTGGG; reverse: TGCCTGTTTCCGTAACCTCAAG (predicted size, 151 bp).

The thermal cycling program applied on the CFX96 real-time system was 95°C for 30 seconds, 40 cycles of 95°C for 5 seconds, and 60°C for 5 seconds, followed by a melting curve analysis to confirm product purity.

Statistical Analysis

Analyses were performed with GraphPad Prism 5 software and presented as median $\pm$ interquartile range. The statistical difference between samples was determined using Kruskal-Wallis test followed by Dunn post hoc test. $P < .05$ was considered significant.

Results

Maternal- and Paternal-Specific Contribution to Placental Development

Numerous studies demonstrate that paternal genes significantly contribute to the development of the placenta and fetal membranes.^{23,24,26,27,29,41} To illustrate the important contribution of the father to these tissues, we utilized male and female mice heterozygous for GFP^{+/+}, resulting in differential GFP expression within the placenta depending on the parental and fetal genotype. As shown in Figure 1A, GFP^{+/+} female mated to a wild-type male results in robust fluorescence within the DZ regardless of whether the fetus is GFP^{+/+} (Figure 1A) or GFP^{-/-} (Figure 1B). However, if the fetus is also GFP^{+/+}, fluorescence is observed within the JZ and LZ zones of the placenta. As expected, the DZ is completely void of fluorescence following the mating of a GFP^{+/+} male to a wild-type female, regardless of the phenotype of the fetus (Figure 1C and D). However, intense fluorescence is noted in the JZ and LZ in the placenta of a GFP^{+/+} pup arising from a GFP^{+/+} father. Also as expected,

Table 1. Dose Response of GBS in Control Pregnancies.

Dose	N	Outcome
Sham	3	Term delivery of healthy pups
10 ⁷	2	Preterm death (E17/18) of dams/pups
10 ⁵	2	Preterm death (E18.5) of dams/pups
10 ⁴	3	Preterm birth (E18.5) death of all pups
10 ²	3	Preterm/early term birth ($\leq$ E19.5) pups survive $\geq$ 24 hours

Abbreviation: GBS, group B *Streptococcus*.

no fluorescence is observed in placenta arising from a GFP^{+/−} male producing a GFP^{−/−} offspring (Figure 1D). Comparing fluorescence observed in Figure 1A and Figure 1C suggests that the father’s contribution to the placenta is largely within the JZ, even beyond the contribution made via his progeny. Although the contribution of the father to the JZ has previously been reported,^{23,25,42,43} the concept that the placental phenotype/genotype can exhibit variation compared to the fetus is not well appreciated.²⁴ In contrast, the fetal membranes are dependent upon contributions of both mother and father,⁴³ and these tissues exhibit fluorescence in pregnancies arising from either GFP^{+/−} mothers or GFP^{+/−} fathers (Supplemental Figure 1). Corresponding hematoxylin and eosin images are shown for each placenta (Figure 1E-H).

Titration of GBS Dose in Control Mice

Our previous studies utilizing lipopolysaccharide (LPS)-induced PTB revealed a much lower dose was needed to induce PTB in our toxicant-exposed female mice (F1_{female}) or in the control partners of F1_{males} compared to control.^{12,44} Therefore, we initially set out to determine the lowest inoculum of GBS which would not induce PTB in control mice (Table 1). Using the clinical GBS strain GB37, we found that all pregnant dams and their pups died within 48 hours of vaginal exposure to 10⁷ CFU or 10⁵ CFU on E15.5. Lowering the inoculum dose to 10⁴ CFU similarly led to 100% pup mortality; however, dams were able to recover. At the lowest feasible dose of GB37 (10² CFU), all control mice still delivered prior to E20, but dams survived and pups were born alive. Therefore, for all remaining experiments, we utilized the 10² CFU inoculum size for GBS.

Pregnancy Outcomes Following Ascending GBS Infection

Following identification of the dose of GBS which was tolerated by control pregnant dams, we subjected an additional group of control dams (N = 9) as well as pregnant F1_{females} (N = 6) and control mating partners of F1_{males} (N = 7) to vaginal inoculation with 10² CFU on E15.5. As expected, gestation length was reduced in all control females mated to control males, with 56% of dams delivering preterm (<E19; Figure 2A). At the inoculation dose of 10² CFU, dams fared well and pups were born alive; however, 19% of pups died within 24 hours of delivery (Figure 2B). Only 50% of control pups survived the first week after birth, but mortality was low thereafter (Supplemental Figure 2). All control females mated

to F1_{males} and exposed to the same ascending GBS infection on E15.5 also delivered prior to E19.5, with 46% delivering preterm (Figure 2A). Mortality of dams was low; however, while pup survival was 70% at 24 hours postdelivery (Figure 2B), only 30% survived to weaning on postnatal day 21 (Supplemental Figure 2). Eighty-three percent of pregnant F1_{females} inoculated with 10² CFU GBS on E15.5 delivered preterm (Figure 2A), while all dams died during parturition or shortly thereafter. Pups were either stillborn (90%) or died within 24 hours of delivery (10%; Figure 2B). For comparison, >95% of control offspring of dams subjected to inoculation with vehicle only survived to weaning, while 80% of F2 offspring (born to either male or female F1 animals) survived to weaning. Although average gestation length in control pregnancies was significantly reduced in the presence of GBS infection (20 days sham vs 18.9 days following GBS exposure, *P* < .001), pregnancy length was not changed in F1_{females} regardless of GBS treatment with an average gestation length of 18.5 in both sham-treated and GBS-exposed animals (*P* = not significant, comparing F1_{females} with and without GBS). Interestingly, partners of F1_{males} exposed to GBS had a slightly longer gestation length compared to partners of F1_{males} treated with vehicle. Specifically, gestation length of sham-treated control partners of F1_{males} was 18.6 days, compared to 18.9 days in GBS exposed (*P* = .03 comparing these 2 groups).

Group B Streptococcus Invasion of Selected Tissues

Additional pregnant mice from all groups (control mating pairs, F1_{females} mated to control males and F1_{males} mated to control females) were subjected to vaginal inoculation with 10² CFU on E15.5 and euthanized 48 hours postinoculation (E17.5). At necropsy, selected maternal tissues were assessed for GBS colonization. Vagina and uteri were swabbed with a sterile Q-tip and samples streaked onto blood agar plates. Maternal blood was diluted 1:10 in PBS and also streaked onto blood agar plates. As shown in Table 2, the vagina of all pregnant animals was positive for GBS colonization, while uterine infection was slightly lower in control females; 100% of F1_{females} mated to control males and control females mated to F1_{males} exhibited uterine infection with GB37. In contrast, the presence of GB37 in maternal blood varied widely between groups, with 57% of F1_{females}, 37.5% of females mated to F1_{males}, and 27% of control females exhibiting the presence of GBS.

Fetal blood from these same dams was also collected at the time of necropsy and the presence of GBS assessed following dilution and streaking onto blood agar plates. As shown in Figure 3, fetal infection rates were quite variable among control pups and in pups of F1_{males}. All pups in each litter were assessed, and each symbol represents the percentage of pups with blood-borne GBS in a single litter. For the control animals, a mean of 36% $\pm$ 11% of pups were GBS positive, while 3 litters were completely free of infection. Among the F1_{male} offspring, 47% $\pm$ 12% of all pups were GBS positive. Two F1_{male} litters had low ($\leq$ 20%) pup infection rates, but no litter was completely free of infection. In contrast, reflecting the

Figure 2. Gestation length and fetal survival at postnatal day 1 (PND1) following GBS infection in late pregnancy. A, Gestation length of control mating pairs, F1_{females} mated to control males and control females mated to F1_{males} and exposed to 10² CFU GBS on E15.5. All animals delivered prior to E19.5; however, F1_{females} inoculated with GBS died during parturition or shortly thereafter. B, Survival rates of offspring were reduced in all groups. Within 24 hours of birth, 19% of control pups had died, while mortality of F1_{male} offspring was 30% on PND1. All pups born to F1_{females} died within 24 hours of birth. **P* < .0001 compared to control. Note that 1 F1_{female} litter was cannibalized prior to 24 hours and was omitted from the graph. GBS indicates group B *Streptococcus*; CFU, colony-forming units.

Table 2. Presence of GBS at Selected Sites 48 Hours Postinoculation.

Mating Pairs	N	Vagina	Uterus	Maternal Blood
CT/CT	9	100%	87.5%	27%
F1 _{Female} /CT	9	100%	100%	57%
CT/F1 _{Male}	8	100%	100%	37.5%

Abbreviation: GBS, group B *Streptococcus*.

100% rate of mortality in pups of F1_{females}, the infection rate of all litters was high (85% ± 9%) with all pups in 3 F1_{female} litters infected with GBS. One litter exhibited a more modest infection rate, with 42% of pups exhibiting blood-borne GBS.

Immunohistochemical Localization of GBS in Placentae

In order to assess the extent of GB37 infection, we next conducted immunohistochemical localization of GBS in placentae from pregnant mice euthanized on E17.5. Consistent with the observations mentioned earlier, placental infection with GBS was minimal in control placentae and in the majority of control/F1_{male} placentae. However, all placentae examined from F1_{female} mice exposed to GBS exhibited some degree of infection, with most exhibiting widespread invasion (Figure 4).

Placental Expression of Tlr2 Messenger RNA and TLR-2 Protein

Toll-like receptor 2 binds GBS and aids in its clearance; therefore, we examined the expression of *Tlr2* messenger RNA (mRNA) and TLR2 protein in placental samples. We expected that F1_{female}-derived placentae would exhibit a loss of this receptor, potentially explaining the extensive colonization of this tissue in these mice. However, as shown in Supplemental Figure 3, *Tlr2* mRNA expression was similar across all groups,

Figure 3. Infection rates in offspring of dams exposed to GBS. Fetal blood was collected on E17.5 from all live pups in each litter and assessed for the presence of GBS. Each symbol represents percentage of infected pups within a single litter. Mean infection rate of control pups was 36% ± 11%, while the mean infection rate of F1_{female} pups was 85% ± 9% and of F1_{male} offspring 47% ± 12% (*P* = .48 compared to control). Note that all pups in 3 litters of F1_{female} mice were dead at the time of maternal necropsy; thus, collection and assessment of fetal blood was not feasible. **P* = .0011. GBS indicates group B *Streptococcus*.

and TLR-2 protein expression also appeared to be similar in placentae of F1_{females} compared to expression observed in tissues from control mating pairs (Figure 5A-B). Surprisingly, placentae derived from F1_{males} exhibit a marked reduction in TLR-2 protein localization (Figure 5C).

Placental Glycogen Production

We previously noted placentae and pups born to F1_{males} were significantly smaller than control pups,¹³ suggesting

Figure 4. Immunohistochemical localization of GBS in E17.5 placentae. Pregnant females were subjected to inoculation with GBS on E15.5 and euthanized 48 hours later. Placental tissues, with decidual tissues attached, were subjected to immunohistochemical localization of GBS, which appears as a brown stain. Control females mated to control males (A), F1_{females} mated to control males (B; $P = .004$), and control females mated to F1_{males} (C). Although GBS could be detected in some control/control and some control/F1_{male} placentae (arrowheads), localization was minimal compared to all placental tissues removed from F1_{females} (D; computer-assisted semiquantitative assessment of staining). Each symbol represents the average staining intensity of all placentae within a single litter. Original magnification, $\times 40$. Inset in A: Negative control. Inset in B, $100\times$, Inset in C, $200\times$. GBS indicates group B *Streptococcus*; DZ, decidual zone; JZ, junctional zone; LZ, labyrinth zone; V: Maternal vasculature.

placental dysfunction which may have compromised fetal nutrition in these pregnancies. Relevant to the current study, placental glycogen is produced by the trophoblast,²⁸ and alterations in glycogen deposition have been associated with fetal growth restriction in human pregnancies.⁴⁵ Importantly, GBS utilizes glucose for proliferation and colonization^{46,47}; therefore, we examined the presence of glycogen-positive cells in E15.5 placentae using PAS staining, with and without diastase treatment, in mice that were not exposed to GBS but corresponding to the timing of bacterial exposure in the experimental animals. As shown in Figure 6, the number of glycogen-positive cells (deep purple) was similar in F1_{female}-derived placentae compared to controls, while placentae from F1_{males} exhibit a significant ($P = .01$) reduction in glycogen-positive cells, which is most apparent within the JZ. These results may further explain the differential response of partners of F1_{males} compared to F1_{females} following GBS infection as well as the previously observed fetal growth restriction in pups of F1_{males}.

Discussion

In the human population, PTB, defined as parturition occurring prior to 37 weeks' gestation, continues to be a major health concern despite decades of intense research. Infants born too soon are at increased risk of death within their first year, while surviving infants can face lifelong disability.^{48,49} Multiple maternal factors, including age, smoking status, and race, are known to contribute to the risk of PTB; however, women with no known risk factors can also deliver early.⁵⁰ Epidemiological studies have demonstrated that paternal factors, such as age and race, can also negatively influence pregnancy outcomes in their partners.⁵¹⁻⁵³ Equally important, epidemiologic studies support a role for environmental exposures in increasing a woman's risk of delivering preterm (reviewed in Stillerman et al⁵⁴). For example, a significant increased risk of PTB was observed in women living within 4 km of a municipal solid waste incinerator,⁵⁵ suggesting exposure to airborne toxicants related to combustion adversely affected pregnancy outcomes. Presumably, the male partners of the women in this study also resided near the incinerator; however, the potential impact of his exposure on PTB

Figure 5. Immunohistochemical localization of TLR2 in E15.5 placentae. Placental and decidual tissues were obtained on E15.5 from mice that were not exposed to GBS but correlating with the timing of bacterial exposure in the experimental mice. Tissues were subjected to immunohistochemical localization of TLR2 (brown staining). Control females mated to control males (A), F1_{females} mated to control males (B), and control females mated to F1_{males} (C). Magnification $\times 40$. TLR2 indicates Toll-like receptor 2; DZ, decidual zone; JZ, junctional zone; LZ, labyrinth zone. Inset in A: Negative control. Computer-assisted semiquantitative assessment of staining is shown in (D). Each symbol represents the average staining intensity of all placentae within a single litter. * $P < .0001$.

was not considered. Although epidemiologic data support the relevance of a man's developmental exposure history to his adult sperm quality,⁵⁶⁻⁵⁸ to our knowledge, no human study has also assessed pregnancy outcomes in his partner.

Important to the current study, several studies have demonstrated early-life exposure to environmental toxicants such as TCDD lead to impaired immune function in adulthood.^{6,7,14,59} Both TCDD and dioxin-like polychlorinated biphenyls act via binding to the aryl hydrocarbon receptor (AhR), an orphan nuclear receptor that has been found to be critical for proper functioning of the immune, hepatic, cardiovascular, vascular, and reproductive systems (reviewed by Mulero-Navarro S and Fernandez-Salguero⁶⁰). Importantly, AhR knockout mice exhibit reduced fertility⁶¹ and immune dysfunction (reviewed by Lawrence and Vorderstrasse⁵⁹). Human and animal data demonstrate developmental exposure to TCDD, and other AhR agonists are associated with a hyperinflammatory response to viral or bacterial infections,^{6,62-64} while our data indicate that such exposures increase the risk of both spontaneous and infection-mediated PTB.¹²

In the absence of other known risk factors, maternal infection with GBS is a significant risk factor for PTB and transmission to the fetus can result in life-threatening disease (reviewed by Vornhagen et al¹⁵). Fetal transmission can occur prenatally via GBS ascension from the vagina or placenta or can occur as a consequence of direct exposure during parturition.^{65,66} Aggressive maternal screening for vaginal-rectal GBS colonization and antibiotic prophylaxis during labor has led to a dramatic reduction in early-onset disease⁶⁵; however, this strategy does not prevent ascending infection prior to term and has not reduced rates of late-onset (postnatal) invasive GBS disease in infants.⁶⁶ Although most babies born to GBS-colonized mothers do not become infected, about 10% of early PTBs can be linked to GBS.¹⁵ Thus, there is a critical need to understand the myriad of factors which influence the progression of GBS infection and lead to adverse outcomes in women and their infants.

Herein, utilizing an established murine model, we examined the influence of a developmental TCDD exposure history of the mother or father on the response to an ascending GBS infection

Figure 6. Identification of glycogen-positive cells in E15.5 placentae using periodic acid–Shiff (PAS) with and without diastase. Placentae were obtained on E15.5 and sister sections subjected to PAS localization of glycogen, which appears as a deep purple stain or PAS staining after diastase treatment to remove glycogen (PAS-D). (A) The PAS-stained placentae from control females mated to control males, placentae from F1_{females} mated to control males, and placentae from control females mated to F1_{males} appear on the left, while PAS staining after diastase appear on the right. Original magnification, 1.25 $\times$. (B) Computer-assisted semiquantitative assessment of staining. * $P < .01$.

during pregnancy. To our knowledge, no previous study has examined a possible influence of the paternal phenotype on maternal GBS infection and fetal outcomes. Given the significant contribution of the father to the placenta and fetal membranes (Figure 1 and the studies by Barton et al, Wang et al, McGrath and Solter, Mitchell et al^{23,24,26,27}), tissues which are important barriers between ascending vaginal infections and the fetus, such an examination is appropriate.

Herein, prior to utilizing our toxicant-exposed mice, we first attempted to identify an inoculum of bacteria that did not disrupt pregnancy in control mice, in an approach similar to that we recently used to examine LPS response in pregnant F1_{females}. For this previous study, we demonstrated that control mice did not exhibit PTB following low-dose LPS (200 μ g/kg) exposure on E15.5, while all F1_{females} delivered within 16 hours of treatment.¹² In an unpublished study, we have also found that partners of F1_{males} similarly exhibit PTB in response to the same low dose of LPS, indicating that the paternal exposure history can also influence infection-mediated PTB. Unfortunately, in the current study, using the highly virulent strain of GBS, GB37,³⁹ we were unable to identify a dose that control animals were completely able to resist (Table 1). However, at an inoculum of 10^2 , control dams fared well and nearly half of their pups survived to weaning (Supplemental Figure 2). Given our previous data demonstrating an enhanced susceptibility of pregnant F1_{females} and partners of F1_{males} to deliver preterm in response to LPS, we anticipated that these animals would also be more sensitive to PTB as a consequence of GBS infection.

However, the deaths of the F1_{females} and all of their pups were unexpected. In retrospect, given that developmental TCDD exposure is known to impair adult immune cell function,⁵⁹ and our data demonstrating loss of the anti-inflammatory action of progesterone in adult F1_{females},^{12,13} it perhaps should not be surprising that these animals succumbed to the infection (Figures 2 and 4). Moreover, maternal and fetal mortality of F1_{females} exposed to 10^2 CFU of GBS was similar to the outcome observed in control females exposed to the 10^5 and 10^7 inocula (Table 1).

In contrast to the F1_{females}, the control partners of F1_{males} actually fared better than expected (Figures 2 and 4). We hypothesized that contributions of the father to the placenta and fetal membranes would promote GBS transmission in pregnancies arising in partners of F1_{males}. However, the reduced expression of TLR2 protein (Figure 5) in concert with the reduction in glycogen content (Figure 6) may have provided the partners of F1_{males} an advantage compared to the F1_{females}. Toll-like receptor 2 is known to play an important role in the immune response to GBS infection, in part as a consequence of inducing a host inflammatory response to limit colonization. Mancuso et al⁶⁷ demonstrated TLR2-deficient mice were unable to clear a low-dose GBS infection, leading to mortality in $\sim 30\%$ of mice. However, this same study found that TLR2-deficient mice fared better than wild-type mice when exposed to a very high dose of GBS, suggesting that death among the wild-type mice was due to development of lethal cytokine storm rather than the bacterial infection per se.

Thus, the reduced TLR2 expression in the placenta of F1_{male} partners may have been advantageous. Additionally, the modest reduction in glycogen-containing trophoblast cells may have also impeded GBS proliferation and survival, as glucose is an important nutrient for this bacterial strain.⁶⁸ Indeed, sequestration of glucose or other nutrients is an important function of nutritional immunity to control the spread of infectious agents, including GBS.^{39,69} Although the reduction in placental glucose in our study appeared modest, the impact of altered glucose levels may have been significant. Specifically, we previously noted that pups of F1_{males} are smaller at birth than age-matched control or F1_{female} offspring.¹³ Since it is the paternally derived trophoblast within the placenta that synthesizes and stores glycogen in order to provide nutrition to the fetus,^{3,28} the modest reduction in glycogen reported herein may also be responsible for the fetal growth restriction previously observed in offspring of F1_{males}. Despite these impediments to GBS within the F1_{male}-derived placentae, offspring of these animals were at a greater risk of infection and mortality compared to control pups. These animals, denoted F2 mice, were also directly exposed to TCDD, since the fetal germ cells are present in the F1 feti at the time of maternal exposure. Thus, impairments in the fetal immune system may also affect GBS transmission and colonization of the pups⁷⁰; however, further studies will be necessary to explore this hypothesis.

In summary, our study indicates that compared to control animals, a developmental exposure history of either parent increases the risk of adverse sequelae in response to an ascending GBS infection. Interestingly, we noted important sex-specific effects that indicate F1_{females} are completely unable to resist infection, resulting in widespread GBS colonization and maternal and fetal death. In contrast, maternal mortality did not occur in partners of F1_{males} similarly exposed to GBS during pregnancy. This dramatically different outcome is likely due to differences in maternal immune responses between the healthy partners of F1_{males} and the F1_{females}, the latter of which have a known hyperinflammatory phenotype.^{12,13} However, perhaps the most interesting observation of the current study was the marked differences noted within the placental phenotypes associated with the sex of the toxicant-exposed parent. The F1_{female}-derived placentae were markedly similar to that of pregnancies arising in unexposed mice; however, placentae derived from partners of F1_{males} exhibit reductions in both TLR2 protein and glycogen stores (Figures 5 and 6).

These observations are likely related. While placentae of control animals are expected to exhibit normal nutritional immunity and sequestration of glucose away from the bacteria, an abnormal production of glycogen by the F1_{male}-derived trophoblasts may have effectively limited bacterial growth in these mice. Consequently, expression of TLR2 protein, normally stimulated by the presence of bacteria,^{71,72} was not induced in the F1_{male}-derived placenta. Alternatively, since TLR2 acts to bind and clear GBS infection via induction of inflammatory signals, based on the data using TLR2 knockout mice described by Mancuso et al,⁶⁷ diminished expression of this receptor may have protected the dams against a lethal

cytokine storm in response to a highly virulent GBS strain. This latter theory is supported by the significant level of infection noted within the offspring of F1_{males} (Figure 2). As discussed previously by Mor and Cardenas, the maternal response to a microbial challenge is dictated not only by her own immune system but also by that of the fetal-placental unit.⁷⁰ Our studies clearly reveal a sex-dependent difference in placental development following early-life toxicant exposure which alters the infection-associated inflammatory response at the maternal-fetal interface. Although extrapolation to humans is speculative at this time, the similarities between the parental origin of the human and mouse placentae suggest that the father should also be considered when attempting to determine a woman's risk of infection-mediated PTB.

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Supplemental Material

Supplementary material for this article is available online.

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