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Developmental 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) Exposure of Either Parent Enhances the Risk of Necrotizing Enterocolitis in Neonatal Mice

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Abstract

Necrotizing enterocolitis (NEC) is a rare, but potentially fatal intestinal inflammatory condition most often arising in premature infants. Infants provided formula are also at greater risk of developing this disease. Although the majority of formula-fed, preterm infants do not develop NEC, up to 30% of infants with the disease do not survive. Thus, identifying additional, currently unrecognized factors, which may predispose a specific infant to NEC development would be a significant clinical advancement. In this regard, we have previously reported that offspring of female or male mice with a history of developmental exposure to the environmental toxicant TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) exhibit altered sensitivity to inflammatory challenges and are frequently born premature. Herein, we examined the possibility that, compared to unexposed mice (F1_{NONE}), developmental TCDD exposure of either parent (maternal, F1M_{TCDD} or paternal, F1P_{TCDD}) would enhance the risk of NEC in offspring (F2_{TCDD} mice) in association with supplemental formula feeding. Beginning on PND7, all neonates were randomized to maternal milk only or maternal milk with up to 20 supplemental formula feedings. All pups remained with the Dams and were additionally allowed to nurse ad libitum. Formula-fed F2_{NONE} pups rarely developed NEC while this disease was common in formula-fed F2M_{TCDD} and F2P_{TCDD} mice. Unexpectedly, 50% of F2M_{TCDD} pups that were not provided supplemental

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formula also developed NEC. Our studies provide evidence that a history of parental TCDD exposure enhances the risk of NEC in offspring and suggest exposure to environmental immunotoxicants such as TCDD may also contribute to this inflammatory disease in humans.

Keywords

Dioxin; Necrotizing Enterocolitis; ileum; lung; multi-generational

Introduction:

Necrotizing enterocolitis (NEC) is a serious and potentially life-threatening neonatal disease that most frequently occurs in premature infants (Gupta & Paria, 2016). NEC develops following bacterial invasion of the intestine, which can lead to loss of the mucosal barrier, inflammation and, in severe cases, bowel destruction (Terrin, Scipione, & De Curtis, 2014; Walker, 2000). It is believed to occur as a consequence of immaturity of the digestive and immune systems (Claud & Walker, 2001) in concert with a lack of diversity within the intestinal microbiome (Dobbler et al., 2017). Additional risk factors for NEC include congenital heart and lung disease and formula-feeding (Isani, Delaplain, Grishin, & Ford, 2018; Kosloske, 1994; Lucas & Cole, 1990). Unfortunately, each of these conditions frequently coexist in preterm infants, leading to the risk of severe disease (Isani et al., 2018; Malhotra, Veldman, & Menahem, 2013).

NEC is estimated to occur in up to 7% of newborns weighing between 500–1500 grams, with 90% of those having been born preterm (Kosloske, 1994; Ou, Courtney, Steinberger, Tecos, & Warner, 2020). As many as 30% of infants weighing less than 1500 grams that develop NEC do not survive (Gephart, McGrath, Effken, & Halpern, 2012; Neu & Walker, 2011) while those that do often face life-long disability and health complications, including short bowel syndrome, liver disease and neurodevelopmental delay (Hunter, Upperman, Ford, & Camerini, 2008; C. R. Martin et al., 2010; Neu, 2014). Despite these grim statistics, it is important to note that majority of preterm infants, even those that are exclusively formula-fed, do not develop NEC, suggesting that a combination of factors ultimately act to determine whether or not an infant will develop this disease. Furthermore, presenting symptoms can be vague and, since NEC is relatively rare, accurate diagnosis may not occur until after significant disease progression (Ou et al., 2020). Thus, identifying additional risk factors that may predispose a specific infant to development of this disease would potentially enable earlier diagnosis and improved intervention strategies.

At the present time, prematurity appears to be the most significant risk factor for NEC. Unfortunately, concomitant with industrialization, the rate of preterm birth (PTB) has risen, suggesting a possible association between parental exposure to pollution and PTB (Candela et al., 2015; Kihal-Talantikite, Zmirou-Navier, Padilla, & Deguen, 2017; Stillerman, Mattison, Giudice, & Woodruff, 2008). In support of this theory, several studies report an increased incidence in premature birth among women residing near solid waste incinerators (Candela et al., 2013; Lin, Li, & Mao, 2006; Santoro et al., 2016) while other studies linked maternal smoking during pregnancy to an increased risk of NEC in their infants (G. Ding et

al., 2017; Downard et al., 2012). Cigarette smoke and incinerator smoke are both known to contain a wide array of toxicants, many of which bind the aryl hydrocarbon receptor (AhR), an orphan nuclear receptor expressed on numerous cells throughout the body (Bock, 2019; Yoshioka & Tohyama, 2019). Inappropriate activation of the AhR during development has been associated with reproductive, immune, neurological and metabolic disorders in adulthood (Bruner-Tran et al., 2017; Guo et al., 2018; Lawrence & Vorderstrasse, 2013; Warner et al., 2020). Among persistent environmental toxicants associated with combustion, TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin, or commonly, dioxin) is known to be a potent an AhR agonist. Lawrence and colleagues have recently shown that developmental exposure to TCDD alters the adult immune response across multiple generations (Post et al., 2019). Importantly, several studies have identified TCDD in human cord blood (Ataniyazova et al., 2001; Morokuma, Tsukimori, Hori, Kato, & Furue, 2017), indicating the occurrence of a developmental exposure with the potential to negatively affect neonatal and adult health (Mallozzi, Bordi, Garo, & Caserta, 2016).

In our previous studies, we examined the multi and transgenerational adult reproductive outcomes in mice with a history of a developmental (F1_{TCDD}/F2_{TCDD}) or ancestral (F3_{TCDD}/F4_{TCDD}) TCDD exposure (Bruner-Tran et al., 2014; Bruner-Tran & Osteen, 2011; T. Ding, Lambert, Aronoff, Osteen, & Bruner-Tran, 2018). These studies revealed that approximately 50% of male and female F1_{TCDD} mice are infertile, while up to 40% of pregnancies arising in these animals end prematurely (Bruner-Tran & Osteen, 2011; T. Ding, McConaha, Boyd, Osteen, & Bruner-Tran, 2011). Additional studies revealed that pregnant F1_{TCDD} females and pregnant partners of F1_{TCDD} males exhibit a heightened response to an inflammatory challenge mediated by viral (T. Ding et al., 2018) or bacterial (Bruner-Tran et al., 2017; Bruner-Tran & Osteen, 2011) exposures as well as stress (Bruner-Tran et al., 2017), leading PTB in 90% of these mice compared to 50% unexposed animals. Furthermore, in each of these studies, we found a persistence of the “inflammatory phenotype” and increased risk of PTB in pregnant F2_{TCDD}/F3_{TCDD} females and pregnant partners of F2_{TCDD}/F3_{TCDD} males. Although the aforementioned studies focused on the *adult reproductive* outcomes, the persistence of TCDD-associated changes across multiple generations suggests that this phenotype is likely present at birth. To test this theory, we subjected unexposed pups (F2_{NONE}) and pups with a paternal TCDD exposure (F2_{P_{TCDD}}) history or maternal exposure (F2_{M_{TCDD}}) history to an inflammatory challenge mediated by formula feeding. We hypothesized that addition of formula to the neonatal diet would induce an inflammatory response in susceptible animals, resulting in the development of NEC.

For the current studies, all offspring of unexposed mice (F2_{NONE}) as well as F2_{M_{TCDD}} and F2_{P_{TCDD}} neonates were allowed to nurse *ad libitum*. At PND 7, pups in each litter were divided between only maternal milk or maternal milk plus supplemental formula feedings. This feeding pattern was designed to emulate the supplemental formula feeding which is common in preterm human infants. Our studies revealed that, compared to F2_{NONE} mice, a maternal history of developmental TCDD exposure significantly increased the risk of spontaneous NEC while exposure of either parent to TCDD was associated with an increased risk of disease following supplemental formula-feeding.

Materials and Methods:

Animals:

Young adult (8–10 weeks) male and female C57BL/6 mice were purchased from Envigo (Indianapolis, IN). Animals were housed in Vanderbilt University's Barrier Animal Care Facility (free of common mouse pathogens) according to National Institutes of Health and institutional guidelines for laboratory animals. Fresh food and water was provided ad libitum. Animal rooms were maintained at a temperature of 22–24°C and a relative humidity of 40–50% on a 12-hour light:dark schedule. Experiments described herein were approved by Vanderbilt University's Institutional Animal Care and Use Committee in accordance with the Animal Welfare Act.

Chemicals:

TCDD (99% in nonane #ED-908) was obtained from Cambridge Isotope Laboratories (Andover, MA). Esbilac® Puppy Milk Replacer Powder was obtained from Pet-Ag, Inc. (Hampshire, IL). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

TCDD Exposure and Mating Scheme:

Virgin C57BL/6 females, aged 10–12 weeks, were mated with intact males of similar age. Upon observation of a vaginal plug, females were separated and denoted as day 0.5 of pregnancy (E0.5). Pregnant mice (F0) were exposed to TCDD (10µg/kg) in corn oil or vehicle alone by gavage at 1100 hours local time on E15.5 (when organogenesis is complete). This *in utero* plus lactational exposure paradigm results in direct exposure of the feti (F1 mice) as well as germ cells which have the potential to become the F2 generation. The selected dose of TCDD reflects the more rapid clearance of this toxicant in mice compared to humans and is well below the LD₅₀ for adult mice of this strain (230µg/kg) (Vogel, Zhao, Wong, Young, & Matsumura, 2003). TCDD given at this time and dose is not overtly teratogenic and gestation length was not affected in the F0 animals; pups (F1 mice) were born on E20 ± 0.5days.

To generate F2 pups for the current study, a single female (F1_{NONE} or F1_{TCDD}) was placed with a single male (F1_{NONE} or F1_{TCDD}) and examined each morning for the presence of a vaginal plug. Animals with a history of in utero TCDD exposure were always mated to unexposed partners, allowing the determination of parent-of-origin effects. Following the identification of a plug (considered E0.5), the male was removed. Females were weighed prior to mating and daily after a vaginal plug was identified. On E16.5, females were examined for signs of pregnancy (weight gain, nipple prominence). Beginning at this time, in order to reduce the risk of cannibalization following the subsequent handling of pups, pregnant females were provided a hut with an exercise wheel (Bio-Serv Mouse Igloo with fast-trac Cat # K3328/K3250). The exposure paradigm, mating scheme and nomenclature of each generation is presented in Suppl Figure 1. Litter size was typically between 4–6 pups per Dam (Tables 1 and 2). Since artificially reducing litter size may lead to overfeeding of remaining pups (see (Suvorov & Vandenberg, 2016), litters were not culled. Equally important, culling of litters may impede the identification of different phenotypes which

may be present within a single litter following a development exposure to an endocrine disrupting chemical (e.g. (T. Ding et al., 2018; Gillette et al., 2017)).

Formula treatment:

Beginning on postnatal day (PND) 7, pups in each litter were sexed and male and female pups randomized to maternal milk only or maternal milk with supplemental puppy formula. Given the small size of litters, this approach typically resulted in 1–2 male and 1–2 female pups in each diet group per litter. All pups in all litters were used and each litter included both treatment regimens. Toenails of pups receiving formula were marked with a small amount of fingernail polish. Pups were bottle-fed 30 uL formula 10–12 times over 4 days using a small nipple attached to a 1mL syringe (Miracle Nipple Mini for Pets and Wildlife). Each 30 uL dose was provided in two 15 uL aliquots ten minutes apart. All pups remained with the Dams and additionally nursed *ad libitum*. Pups were monitored daily for overt signs of intestinal necrosis, including abdominal distension, feeding intolerance, rectal bleeding, lethargy and apnea. Pups receiving 10–12 doses of formula were euthanized on PND11 (24 hrs after the last supplemental feeding). Littermates that were exclusively nursed were also euthanized on PND11.

A second series of studies was conducted in which pups were provided extended formula supplementation. These studies were largely conducted as described above, except that pups were provided 20 doses of formula over two weeks, beginning on PND7. These pups, along with littermates not receiving supplementation, were euthanized on PND21.

Euthanasia and collection of tissues:

On PND11 or PND21, pups were euthanized by decapitation performed under deep anesthesia per AAALAC guidelines. After euthanasia, the peritoneal cavity was opened and visually inspected for the presence of edema. The intestine was then removed from proximal duodenum to terminal colon. Tissues were gently flushed to remove fecal material. A small segment of the large intestine was frozen for RNA analysis. The remaining intestine was wrapped using the “swiss-roll technique” (Park, Reid, Walker, & MacPherson, 1987; Whittem, Williams, & Williams, 2010) and tissues examined macroscopically for signs of inflammation (Figure 1A–B). Lungs were removed from a subset of animals, perfused and fixed with formalin for histologic evaluation. All fixed samples were processed, paraffin-embedded and slides containing multiple 4 um sections prepared by the Vanderbilt Translational Pathology Shared Resource (TPSR) using standard methodology.

Histologic Scoring:

Intestinal tissues were subjected to hematoxylin and eosin staining by standard methods. Blinded samples were independently analyzed by three investigators. The presence and grade of NEC (Figure 2) was determined using the criteria adapted from the histologic injury scoring system previously described by others (Caplan, Hedlund, Adler, & Hsueh, 1994; Ran-Ressler et al., 2011). Briefly, Grade 0: intact villi; Grade 1: superficial epithelial cell sloughing with mild, intermittent separation of the submucosa; Grade 2: moderate to severe separation of the submucosa with villous sloughing; Grade 3: severe separation of the submucosa and/or lamina propria region, villous sloughing and initial villus necrosis; Grade

4: transmural necrosis and/or loss of villi structure. Independent analyses were then compiled for final stage determination. In the event the reviewers were not in agreement, the majority opinion was used. A histologic injury score of 2 is consistent with the presence of NEC (Caplan et al., 1994; Ran-Ressler et al., 2011).

Immunohistochemistry:

Immunohistochemical staining was performed by Vanderbilt's TPSR core laboratory using a commercially available antibodies as described below using the Leica Bond Max automated processor for immunostaining. All steps besides dehydration, clearing and coverslipping were performed on the Bond For all antibodies, heat induced antigen retrieval was performed using the Bond Max Epitope Retrieval 2 solution for 20 minutes. For each study, slides to be stained were selected at random from all available animals. For F2_{NONE} groups, multiple pups were selected from a minimum of three litters, while a minimum of five litters were used for F2_{TCDD} pups.

Macrophages—Deparaffinized and rehydrated tissues were incubated with anti-F4/80 (NB600–404, Novus Biologicals LLC, Littleton, CO) for one hour at a 1:900 dilution and then incubated in a rabbit anti-rat secondary (BA-4001, Vector Laboratories, Inc., Burlingame, CA) for 15mins at a 1:2000 dilution. The Bond Polymer Refine detection system was used for visualization. Slides were then dehydrated, cleared and coverslipped. Semi-quantitative assessment of macrophages was conducted by assessing staining intensity of macrophage-associated chromogen using the methodology previously described (Nguyen, Zhou, Shu, & Mao, 2013). Although the color of the chromogen is darker in regions with more antigen, the darker staining results in a lower intensity value. Thus, staining is reported by calculating the “reciprocal intensity” by subtracting the intensity of the stained area of interest from the maximum (no staining). Herein, multiple nonoverlapping fields (20x magnification) were selected and analyzed for each animal with a minimum of 5 pups per group from a minimum of three different litters per group. Images were captured using an Olympus (Tokyo, Japan) BX51 microscope system and Olympus DP71 digital camera. Staining intensity was assessed using Fiji software (ImageJ; Rockville, MD) and reciprocal intensity 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).

Proliferation and Vascularization: Proliferation and vascularization in ilea was assessed by dual staining using antibodies against Ki67 and CD31, respectively. Briefly, deparaffinized and rehydrated tissues were incubated with rat anti-CD31 (Catalog #DIA-310, Dianova, Hamburg, Germany) for one hour at a 1:30 dilution followed by biotinylated anti-rat (Cat.# BA-4000, Vector Laboratories, Inc., Burlingame, CA) for 15 minutes at a 1:2000 dilution. The Bond Refine (DS9800, Buffalo Grove, IL, USA) detection system was used for visualization. The sections were then incubated with rabbit anti-Ki67 (Catalog #12202S, Cell Signaling Technology, Danvers, MA) diluted 1:200 for one hour. The Bond Polymer Refine Red Detection system (cat#DS9390, Leica Biosystems, Newcastle Upon Tyne, United Kingdom) was used for visualization. Finally, slides were dehydrated, cleared and coverslipped. Photomicrographs were taken using the same Olympus system described above.

Quantitative RT-PCR analysis of *Tlr4*:

Total RNA was isolated from the proximal colon with Trizol (Invitrogen, Carlsbad, CA) and purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA from 1 µg of total RNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad) and random decamer primers. Primers (forward and reverse) for *Tlr4* were obtained from Sino Biological (Cat#MP200636). The thermal cycling program applied on the CFX96 Real-time System was: 95°C for 30 s, 40 cycles of 95 °C for 5 s, 60 °C for 5 s, followed by a melting curve analysis to confirm product purity. Reactions were performed in triplicate in a Bio-Rad CFX96 Real-time thermocycler system. *Ribosomal 18s* was used as an endogenous control for all samples. Results were evaluated using the delta-delta Ct method as previously described (T. Ding et al., 2011; McConaha et al., 2011).

Statistical Analysis:

Analyses were performed with GraphPad Prism©5 software and presented as mean ±SEM. The statistical difference between two samples was determined using Student's t test while comparison between multiple samples/treatment groups were analyzed using Kruskal-Wallis one-way analysis of variance (one-way ANOVA on ranks) followed by Dunn's post-hoc test. Since we and others have reported phenotypic differences between individual pups in a single litter following exposure to an environmental toxicant (T. Ding et al., 2018; Suvorov & Vandenberg, 2016), for these studies, all pups in each litter were used and divided between treatment groups. Thus, the pup was used as the statistical unit and multiple litters included. $P < 0.05$ were considered significant.

Results:

Macroscopic and Microscopic Assessment of Intestine

All pups exclusively nursed or provided only short-term formula supplementation appeared to fare well throughout the treatment period with no mortality and no external signs of NEC, regardless of TCDD exposure history. Microscopic assessment of ilea from F2_{NONE} and F2P_{TCDD} pups that were exclusively nursed revealed normal histology in all animals (Figure 2). In contrast, 50% of F2M_{TCDD} pups receiving *only maternal milk* developed NEC. Following short-term formula, mild NEC was noted in 12.5% of F2_{NONE} pups, while 75% of F2M_{TCDD} pups and 80% of F2P_{TCDD} pups developed Grade 2 NEC (Figure 2).

A second cohort of pups was provided an extended supplemental formula exposure and examined for NEC. F2_{NONE}, F2M_{TCDD} and F2P_{TCDD} pups were randomized on PND7 to exclusive nursing or nursing plus 20 doses of formula over a period of 2 weeks. At the time of necropsy on PND21, all F2_{NONE} pups provided only maternal milk appeared healthy on gross exam and microscopic exam revealed an absence of NEC (Figure 2). However, microscopic assessment of pups with a parental TCDD exposure revealed NEC in two of twelve (17%) F2P_{TCDD} and six of ten (60%) F2M_{TCDD} pups (Figure 2). Following prolonged formula supplementation, one of thirteen (8%) F2_{NONE} pups was found to have intestinal edema and rectal bleeding, while nine of twelve F2P_{TCDD} (75%) pups subjected to prolonged formula feeding exhibited rectal bleeding at necropsy ($p < 0.001$, compared to F2_{NONE}). Among F2M_{TCDD} pups receiving prolonged formula, five of thirteen (38%) mice

exhibited mild rectal bleeding at the time of necropsy ($p=0.069$ compared to F2_{NONE}). Microscopic examination of the ileum revealed that the majority of F2_{NONE} pups receiving prolonged formula developed NEC, with 50% exhibiting Grade 2 disease and 17% exhibiting Grade 3 disease (Figure 2). However, *all* F2P_{TCDD} pups provided prolonged formula treatment were identified as having NEC, with 80% exhibiting severe (Grade 3 or 4) disease (Figure 2). Among F2M_{TCDD} pups treated with 20 doses of formula, 77% developed NEC with five of thirteen (38%) developing severe disease (Figure 2).

Intestinal Cell Proliferation and Vascularization

NEC in both human infants and in animal models of the disease have revealed reduced intestinal proliferation in response to injury as well as impaired vascularization (Bowker, Yan, & De Plaen, 2018; Li et al., 2019; Pisano et al., 2020; Yan et al., 2016). Therefore, we assessed cell proliferation and vascularization within the intestine using immunohistochemical localization of antibodies against Ki67 and CD31, respectively. In F2_{NONE} pups receiving either maternal milk only or short-term formula supplementation, Ki67 was observed along the crypts, continuous with the base of the villi (pink staining, Figure 3A,D). This pattern was consistent with that of control intestinal tissue as described in the literature (Erben et al., 2014; Ran-Ressler et al., 2011). Short-term formula-feeding of F2_{NONE} pups was associated with a modest increase in localization of Ki67, which is likely a repair associated response to an inflammatory challenge (Wong & Wright, 1999). Examination of Ki67 localization in F2M_{TCDD} pups that were exclusively nursed or provided short-term formula supplementation revealed a similar staining pattern as observed in F2_{NONE} mice (Figure 3C,F). Interestingly, compared to F2_{NONE} and F2M_{TCDD} pups, Ki67 staining was lower in all F2P_{TCDD} pups regardless of diet (Figure 3B,E).

Assessment of vascularization within the same samples revealed CD31 localization to endothelial cells, which were most abundant within the villi (brown staining, Figure 3). Compared to F2_{NONE} and F2M_{TCDD} pups receiving only maternal milk, untreated F2P_{TCDD} pups consistently exhibited a reduction in CD31 staining (Figure 3B). Interestingly, CD31 staining of intestinal villi was increased in F2_{NONE} and F2M_{TCDD} pups treated with short-term formula (Figure 3D,F), while localization of this protein was diminished in all F2P_{TCDD} pups receiving short-term formula (Figure 3E).

A similar assessment of Ki67 and CD31 was conducted in intestinal tissues obtained from pups provided extended formula (Figure 3G–I). Staining patterns of both proteins varied and appeared to be dependent upon the extent of histologic injury. Specifically, all pups developing NEC as a consequence of 20 doses of formula exhibited decreased immunolocalization of CD31 (Figure 3J–L), indicating reduced vascularization; however, reduced proliferation, as assessed by Ki67 staining, was only observed in tissues from F2P_{TCDD} pups (Figure 3K).

Localization of Macrophages

Development of NEC has been linked to alterations in the innate immune response, most likely as a consequence of immunologic immaturity (Maheshwari, 2015). In particular, increased numbers of macrophages have been identified in human and experimental NEC

and inflammatory cytokines produced by these cells likely contribute to the development of disease (Maheshwari, 2015; MohanKumar et al., 2012). Therefore, ileal samples from PND11 and PND21 pups were subjected to F4/80 staining to assess the presence of macrophages. As shown in Figure 4 F2_{NONE} ilea exhibited a non-significant increased localization of macrophages in response to short and extended formula treatment compared to littermates that were exclusively nursed. Unexpectedly, localization of macrophages to ilea of F2P_{TCDD} and F2M_{TCDD} pups was highly variable and appeared to be independent of diet as well as disease state (Figure 4). Several studies have reported multi and transgenerational alterations in immune system development as a consequence of developmental or ancestral TCDD exposure (Post et al., 2019; Vorderstrasse, Cundiff, & Lawrence, 2004). Thus, the variable macrophage response of our F2P_{TCDD} and F2M_{TCDD} pups may reflect similar immune impairments due to their parental TCDD exposure. Alternatively, it is also possible that macrophages were lost in association with tissue destruction following the development of NEC, resulting in an artificial decrease in cell number.

***Tlr4* mRNA Expression**

Expression of toll-like receptor-4 (TLR4), a pattern recognition receptor, has previously been found to be increased in intestinal tissues of infants with NEC (reviewed in (Mihi & Good, 2019). Herein, we examined *Tlr4* mRNA expression in the colon of F2_{NONE}, F2P_{TCDD} and F2M_{TCDD} pups exclusively nursed or additionally provided short-term formula. Although expression of *Tlr4* appeared to be slightly increased in mice with a parental history of TCDD exposure compared to F2_{NONE}, these changes only reached significance in F2M_{TCDD} pups provided formula compared to formula supplemented F2_{NONE} pups (Figure 5).

Lung Dysplasia as a Consequence of TCDD-Associated NEC

Lung dysplasia, potentially leading to episodes of hypoxia, is a common co-morbidity in human infants with NEC (R. J. Martin, Di Fiore, & Walsh, 2015). Therefore, we examined the pulmonary histology in a subset of F2_{NONE}, F2M_{TCDD} and F2P_{TCDD} pups. Formalin-fixed tissues from pups exclusively nursed or additionally provided short-term formula were subjected to standard hematoxylin and eosin staining and microscopic examination. Although lungs of PND11 F2_{NONE} and F2P_{TCDD} pups provided only maternal milk appeared healthy (Figure 6A–B), lungs of F2M_{TCDD} pups provided only maternal milk varied in appearance. Specifically, F2M_{TCDD} pups provided only maternal milk that did not exhibit intestinal signs of NEC exhibited normal pulmonary histology or only mild inflammation (Figure 6C). However, pulmonary dysplasia, characterized by airspace destruction, was observed in nursing only F2M_{TCDD} pups that developed spontaneous NEC (Figure 6D). Following short-term formula, all animals examined exhibited a reduction in airspace, which was most severe in pups with a history of parental TCDD exposure (Figure 6E–G).

Discussion:

NEC is a serious gastrointestinal disease that most frequently affects preterm and low birth weight neonates and appears to be related to immaturity of the intestinal and immune defense systems (Isani et al., 2018; Malhotra et al., 2013). The estimated economic burden of NEC is approximately \$5 billion per year in the United States alone, accounting for 20% of neonatal care expenditures (Gephart et al., 2012). Although advancing medical and surgical practices have improved treatment and survival outcomes, predicting at-risk infants and clinical interventions for prevention of NEC in this population remain under-researched (Patel, Panagos, & Silvestri, 2017). Unfortunately, identifying effective prevention measures is limited by an incomplete understanding of disease etiology as well as the clinical observation that only a fraction of at-risk infants ultimately develops NEC. Clearly, additional, currently unknown risk factors, also contribute to disease development in a subset of infants. Several studies have examined the potential contribution of maternal smoking to the risk of NEC (G. Ding et al., 2017; Downard et al., 2012; Myles, Espinoza, Meyer, Bieniarz, & Nguyen, 1998; Yusuf et al., 2018); however, these studies did not consider the father's smoking status.

In order to directly examine the potential role of either a maternal or paternal history of environmental toxicant exposure on development of NEC in offspring; we utilized a well-established model of in utero TCDD exposure. TCDD is a ubiquitous environmental toxicant and multiple studies have found that in utero exposure negatively impacts immune system development (Lawrence & Vorderstrasse, 2013). For example, Post et al (Post et al., 2019) reported adult mice with a direct or ancestral history of TCDD exposure exhibited an impaired ability to clear influenza infection while Peltier et al reported similarly exposed mice exhibit an inflammatory phenotype during pregnancy (Peltier et al., 2013). Our previous studies demonstrated that adult male and female F1_{TCDD} animals consistently demonstrate a heightened inflammatory response to either viral and bacterial challenges (T. Ding et al., 2018; T. Ding et al., 2011). Importantly, offspring of either F1_{females} (F2_{M_{TCDD}}) or F1_{males} (F2_{P_{TCDD}}) are frequently born preterm (T. Ding et al., 2011) and exhibit a higher neonatal mortality rate compared to unexposed neonates (T. Ding et al., 2018). These data suggested to us that neonatal mice with a parental exposure to TCDD would also be more sensitive to other exogenous inflammatory stressors. We theorized that supplemental formula feeding, acting as such a stressor, would promote the development of NEC in these mice.

Our studies revealed that, compared to unexposed neonates, F2_{P_{TCDD}} pups exhibit an enhanced susceptibility of formula-associated NEC while F2_{M_{TCDD}} pups frequently developed spontaneous as well as formula-associated disease. Following long-term formula supplementation, both F2_{M_{TCDD}} and F2_{P_{TCDD}} pups were more likely to exhibit bloody stool at necropsy as well as more severe disease on microscopic examination (Figure 2). Proliferation was reduced in all pups with a history of TCDD exposure while F2_{P_{TCDD}} pups also exhibited impaired vascularization (Figure 3). Although F2_{NONE} pups demonstrated a robust macrophage infiltrate in response to formula, F2_{P_{TCDD}} and F2_{M_{TCDD}} pups did not exhibit a predictable immune trafficking response (Figure 4). As stated above, this data is perhaps not surprising, given that developmental exposure to TCDD and similar toxicants

have previously been found to disrupt ontogeny of both the innate and adaptive the immune system. Depending on the compound, dose and timing, consequences of developmental exposure have been found to result in immunosuppression or immunostimulation in adulthood (Boule et al., 2015; Boule, Winans, & Lawrence, 2014; Bruner-Tran et al., 2017; Lawrence & Vorderstrasse, 2013; Pajewska-Szmyt, Sinkiewicz-Darol, & Gadzala-Kopciuch, 2019). Furthermore, several lines of evidence demonstrate phenotypic differences in NEC-macrophages compared to cells from healthy infants. Specifically, NEC-associated macrophages exhibit a hyperinflammatory response, which likely promotes tissue damage in association with this disease (reviewed by (Maheshwari, 2015). Thus, despite the reduced number of macrophages observed in F2_{TCDD} mice, cells that are present may exhibit a lower threshold for activation or a heightened cytokine response following activation (Okazaki, Nishida, & Kimura, 2016).

Activation of immune cells occurs following the binding of toll-like receptors (TLR) to pathogen-associated molecular patterns (PAMPs) that are expressed on infectious agents. Increased intestinal expression of *Tlr4* has been described in preterm human infants as well as in experimental models of NEC (Mihi & Good, 2019). Therefore, we assessed expression of this gene in tissues from F2_{NONE} and F2_{TCDD} pups, with and without short-term formula. Although a significant increase in *Tlr4* mRNA expression was only observed in formula fed F2_{TCDD} pups compared to other groups (Figure 5), our study was limited by the collection of only a single colon sample for RNA analysis. NEC, even in severe forms, rarely involves the entire intestine and is frequently patchy in its presentation. Since the majority of each intestine was fixed, histologic assessments were conducted over large areas; however, samples collected for *Tlr4* mRNA analysis may not have been affected even if the animal exhibited NEC in other areas of the intestine. Analysis of additional samples that include both affected and unaffected areas is warranted in future studies.

Taken together, data presented herein strongly suggest that a parental history of developmental TCDD exposure impacts the offspring risk of NEC. Interestingly, F2_{TCDD} offspring in all treatment groups exhibited a more variable occurrence of NEC compared to F2_{TCDD} neonates. Unexpectedly, half of the F2_{TCDD} pups exclusively nursed were found to develop NEC. Spontaneous NEC was not observed in the F2_{NONE} pups and was only rarely identified in F2_{TCDD} mice (Figure 2). Although addition of formula to the F2_{TCDD} pup diet increased the incidence of NEC, incidence and severity of formula-associated disease was greatest in F2_{TCDD} pups. These results suggest a divergence of the impact of a maternal versus paternal history of TCDD exposure on the neonatal inflammatory response to an exogenous challenge, such as formula-feeding.

There is compelling evidence for the role of formula feeding in the development of both inflammatory colitis and NEC in humans and experimental models (Bergholz et al., 2013; Good, Sodhi, & Hackam, 2014; Ramani & Ambalavanan, 2013). In human infants, NEC was 6–10 times more common in premature infants exclusively provided formula compared to those exclusively breastfed (Lucas & Cole, 1990). For this reason, it is widely believed that breastmilk plays a protective role against NEC in a dose-dependent fashion (Good et al., 2014). For example, breastmilk IgA has recently been suggested to play a significant role in establishing a healthy neonatal gut microbiota and reducing the risk of NEC (Gopalakrishna

et al., 2019). Although it is currently not known whether a developmental exposure to TCDD would impact breastmilk IgA, Kinoshita et al (Kinoshita et al., 2006) reported that acute exposure to this toxicant impaired IgA production in the murine gut. Thus, Dams with a history of in utero TCDD exposure may exhibit altered milk composition while pups may have impaired gut IgA, further compounding the intestinal changes reported herein.

In summary, our study indicates that either a maternal or paternal history of developmental TCDD exposure plays a significant role in increasing the risk for NEC in offspring. Importantly, the disease developing in our model exhibits numerous similarities with human NEC, including the development of pulmonary dysplasia (Figure 6). Despite advancements in biomedical research and neonatal care, the overall survival rate of NEC has not markedly improved (Bergholz et al., 2013). Our studies suggest that the environmental exposure history of the parents may be an important “missing link” in identifying at risk infants that are more likely to progress to life-threatening NEC. Developing a better understanding of the mechanisms by which paternal and/or maternal exposure to TCDD, and possibly other toxicants with similar bioactivity, impacts development of NEC in newborns should provide insight into identifying strategies to predict and/or prevent this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: Gross Assessment at Necropsy:
Swiss roll presentation of (A) Normal intestine and (B) Intestine exhibiting inflammation (arrows).

Figure 2: Scoring and Incidence of NEC:

Microscopic assessment of histological injury was determined by an established grading system. Hematoxylin and eosin staining of ileum from pups exhibiting normal intestinal architecture, Grade 0; superficial epithelial cell sloughing with mild, intermittent separation of the submucosa, Grade 1; moderate to severe separation of the submucosa with villous sloughing, Grade 2; severe separation of the submucosa and/or lamina propria region, villous sloughing and initial villus necrosis, Grade 3 and transmural necrosis and/or loss of villi structure, Grade 4. A histologic injury score of ≥ 2 is consistent with the presence of NEC. Original magnification, 20x.

Scatterplots of the histologic injury score from all pups provided 10–12 doses of formula (left panel) or 20 doses of formula (right panel) are shown. For this set of experiments, treatment groups consisted of pups from 4 different litters, with each litter divided between exclusive nursing and formula supplementation. Across all treatment groups, a parental history of TCDD exposure was associated with a significantly increased risk of developing NEC compared to unexposed pups ($p < 0.001$). Within each exposure group, only F2P_{TCDD} pups demonstrated a significant difference in NEC risk between nursing only and both short-

term and extended formula supplementation ($p<0.001$). F2_{NONE} pups exhibited a modest, but significant ($p=0.01$) increased risk of NEC following extended formula compared to maternal milk only. No significant differences in incidence of NEC were observed between F2M_{TCDD} pups that were exclusively nursed versus those provided any amount of supplemental formula. For both series of studies, we did not identify a difference in the development of NEC between male and female pups (data not shown). Therefore, data for both sexes is presented collectively.

Figure 3. Assessment of Proliferation and Vascularization of Ileal Tissue from F₂NONE, F₂P_{TCDD} and F₂M_{TCDD}:

All tissues were subjected to Ki67 (pink) and CD31 (brown) immunolocalization. Representative sections of ileal tissue from F₂NONE (A), F₂P_{TCDD} (B) and F₂M_{TCDD} (C) exclusively nursed, F₂NONE (D), F₂P_{TCDD} (E) and F₂M_{TCDD} (F) pups provided 10–12 doses formula and F₂NONE (G), F₂P_{TCDD} (H) and F₂M_{TCDD} (I) pups provided 20 doses of formula are shown. Original magnification, 20x. For this set of experiments, treatment groups consisted of pups from 4 different litters with all litters divided between nursing only and formula supplementation.

Figure 4: Immunolocalization of Macrophages in Ileal tissues from F2_{NONE}, F2P_{TCDD} and F2M_{TCDD}: Ileal tissues from all groups were subjected to immunolocalization of macrophages using an antibody to F4/80 (brown staining). Representative sections of ilea from F2_{NONE} (A), F2P_{TCDD} (B) and F2M_{TCDD} (C-D) pups exclusively nursed, F2_{NONE} (D), F2P_{TCDD} (E) and F2M_{TCDD} (F) pups provided 10 doses formula and F2_{NONE} (G), F2P_{TCDD} (H) and F2M_{TCDD} (I) pups provided 20 doses of formula are shown. Original magnification, 20x. Semi-quantitative analysis of staining from all pups is shown in J, with the y-axis depicting the reciprocal intensity value and the x-axis the number of doses of formula within each exposure group. Each symbol represents the average reciprocal intensity value for multiple images from one individual animal. For this set of experiments, multiple nonoverlapping fields (20x magnification) were selected and analyzed for each animal with a minimum of 5 pups per group selected from 4 different litters per group. Pups in all litters were divided between maternal milk only and formula supplementation.

Figure 5. *Tlr4* mRNA Expression:

Intestinal RNA was isolated from F2_{NONE}, F2_{P-TCDD} and F2_{M-TCDD} pups provided maternal milk only or short-term formula supplementation and subjected to qRT-PCR for *Tlr4*. For this set of experiments, each exposure and treatment group consisted of 6 pups from multiple litters. *Tlr4* mRNA expression was significantly increased in tissues from F2_{M-TCDD} pups compared to F2_{NONE} (** $p < 0.01$). MM, Maternal Milk; Form, Formula.

Figure 6: Assessment of Lung Dysplasia in F2_{NONE}, F2P_{TCDD} and F2M_{TCDD}: Hematoxylin and Eosin staining of lung tissue from F2_{NONE} (A), F2P_{TCDD} (B) and F2M_{TCDD} (C-D) pups provided exclusively nursed or F2_{NONE} (E), F2P_{TCDD} (F) and F2M_{TCDD} (G) pups short-term formula supplementation. Original magnification, 20x.

Table 1:

Pregnancy Rate, Litter Sizes and Pup Distribution (Short-term Formula Study)

F1 Maternal (N)	F1 Paternal (N)	%Fertile* (N of litters)	Total Pups	Avg Litter Size**	F2 Diet	
					Maternal Milk Only (N)	10–12 Doses Formula (N)
F1 _{NONE} (5)	F1 _{NONE} (5)	100% (5)	26	5.2	10	16
F1 _{NONE} (15)	F1 _{TCDD} (15)	53% (8)	31	3.9	14	17
F1 _{TCDD} (13)	F1 _{NONE} (13)	46% (6)	22	3.7	10	12

* Mice were considered infertile after identification of 4 vaginal plugs without a resulting pregnancy.

** Pups that died prior to randomization were excluded.

Table 2:

Pregnancy Rate, Litter Sizes and Pup Distribution (Extended Formula Study)

F1 Maternal (N)	F1 Paternal (N)	%Fertile* (N of litters)	Total Pups	Avg litter size**	F2 Diet	
					Maternal Milk Only (N)	20 Doses Formula (N)
F1 _{NONE} (4)	F1 _{NONE} (4)	100% (4)	25	6.25	12	13
F1 _{NONE} (12)	F1 _{TCDD} (12)	50% (6)	24	4.0	12	12
F1 _{TCDD} (15)	F1 _{NONE} (15)	40% (6)	23	3.83	10	13

* Mice were considered infertile after identification of 4 vaginal plugs without a resulting pregnancy.

** Pups that died prior to randomization were excluded.