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Maternal treatment with a high dose of CpG ODN during gestation alters fetal craniofacial and distal limb development in C57BL/6 mice ☆

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Abstract

Synthetic oligodeoxynucleotides (ODN) containing CpG motifs, characteristic of bacterial DNA, are currently being evaluated as vaccine adjuvants for inducing protective immunity. Recently, there is increasing pressure to vaccinate pregnant women against maternally transmitted diseases including AIDS and tetanus, as well as against potential bio-weapons such as anthrax. CpG vaccines are effective because they trigger transient increases in T_H1 cytokine production. Recent literature suggests, however, that a shift toward a T_H1 cytokine profile during pregnancy may increase the risk of fetal morphologic defects. On this basis, we hypothesized that exposure to CpG motifs during pregnancy could result in T_H1 inflammation leading to adverse effects on fetal development. To address this hypothesis, pregnant C57BL/6 mice were injected with CpG ODN (0–300 μg/dam) and maternal and fetal outcomes were determined. Injection of dams with the highest dose of CpG ODN resulted in markedly increased fetal resorptions and craniofacial/limb defects, while lower doses had little, if any effects. Histological examination of placentas revealed cellular necrosis with mixed inflammation and calcification in the spongiotrophoblast layer and dysregulation of labyrinthine vascular development. Concomitant elevations in maternal serum cytokine levels were observed including interleukin (IL)-2, IL-10 and IL-12. Treatment with 300 μg of non-CpG ODN did not cause any adverse effects. The 300 μg dose of CpG ODN used in the present study is 30-fold higher than the highest dose that has been administered to humans during clinical trials. These results suggest that the induction of T_H1 cytokines during pregnancy by CpG motifs may potentially increase the risk of fetal loss and morphologic defects in mice, at least at high doses, and support the need for further investigation of teratogenesis that may result from exposure to vaccine adjuvants designed to produce T_H1 cytokine profile shifts.

Keywords: CpG ODN; Immunostimulatory DNA; Fetal malformation; Craniofacial and distal limb development; Abortion; Th1 cytokine shift

1. Introduction

The Toll-like receptor (TLR) family recognizes specific pathogen-associated molecular patterns such as bacterial lipopolysaccharides (LPS) and bacterial DNA. Activation of immune cells though TLR binding produces a myriad of cytokines and chemokines important in orchestrating

☆ The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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immune responses [1,2]. TLR-9 recognizes bacterial DNA through unmethylated CpG motifs (unmethylated deoxycytidyl-deoxyguanosin dinucleotides) that are present in much higher frequency in prokaryotic DNA than eukaryotic DNA [2–5]. Synthetic oligonucleotides (ODNs) containing unmethylated CpG motifs are capable of recapitulating the immune response to bacterial DNA, and as such their potential immunotherapeutic uses have been the focus of intensive research (reviewed in [6]). Potential therapeutic uses include activation of protective immunity [7,8], asthma immunotherapy [9,10], cancer therapy [11,12] and improvement of vaccine efficacy [13].

Maternal vaccination has been proposed as a means to provide neonates and young infants with sufficient immunity to resist potentially fatal infections [14]. As such, new vaccine technologies could result in fetal exposure to CpG motifs present in plasmid DNA vaccines and also directly through the use of CpG ODNs as vaccine adjuvants. In addition, significant workplace exposure to bacterial products occurs in numerous occupations where pregnant women are present including farming [15], laboratory animal care [16] and metal fabrication where exposure to microbial contaminated metalworking fluids occurs [17]. Occupational exposure could also result from vaccination of military personnel with plasmid vaccines or CpG adjuvant vaccines directed against bioterrorism agents including anthrax. In addition, animal studies have demonstrated transplacental transfer of DNA following maternal exposure via oral [18] and parenteral routes [19,20], suggesting the possibility for fetal exposure to foreign DNA subsequent to therapeutic use and environmental/occupational exposure. As such, safety concerns exist regarding therapeutic uses of CpG ODNs and/or CpG DNA exposure during pregnancy. Since interaction of CpG motif-rich bacterial DNA and synthetic ODNs with TLR-9 initiates a signaling cascade culminating in immune cell activation and increased production of predominantly inflammatory cytokines including IL-12, IFNγ, IL-2, and TNFα, therapeutic or occupational exposure to CpG motifs during pregnancy could potentially produce deleterious effects on maternal-fetal health. The present studies were conducted to determine the effects of maternal treatment with CpG ODN during gestation on fetal survival and development.

2. Materials and methods

2.1. CpG oligonucleotides

ODNs were synthesized by TriLink Biotechnologies (San Diego, CA) using an endonuclease-resistant phosphorothioate backbone, which extends the half-life and activity of CpG ODNs in vivo [5,21]. The immunostimulatory ODN (ODN 1826), hereafter referred to as CpG ODN, has the sequence 5′-TCCATGA*CG*TTCCTGA*CG*TT-3′ with the CpG motifs underlined. The control ODN, hereafter referred to as non-CpG ODN, has the sequence 5′-TCCATGA*GC*TTCCTGA*GTC*T-3′ where the CpG motifs have been rearranged resulting in an alteration of immunostimulatory response towards T_H2 immunity [22]. ODNs were reconstituted in pyrogen-free phosphate buffered saline (PBS) at 10 mg/ml and subsequently analyzed for LPS content, which was <0.05 EU/mg as tested using Endosafe® (Charles River Laboratories, Charleston, SC).

2.2. Animals and treatment

Male and female specific-pathogen-free C57BL/6 mice were obtained at 6 weeks of age from Jackson Laboratories (Bar Harbor, ME). Males were housed individually and females in groups of three per cage for an acclimatization period of 2 weeks. A microisolator caging system was used to maintain the specific-pathogen-free status of the animals and prevent exposure to microbes. Food and water were supplied ad libitum and the animals were maintained in the AAALAC accredited NIOSH animal facility under controlled conditions (21 ± 2 °C and 50 ± 10% relative humidity) using a 12 h light/dark cycle. All studies were conducted using protocols (02-ML-M-013, 03-VJ-M-013) approved by the Institutional Animal Care and Use Committee under the guidelines of the Public Health Services Policy on Humane Care and Use of Laboratory Animals. Two study designs that were conducted in parallel were used to examine the effects of gestational treatment with CpG ODN on fetal malformation (design #1) and pregnancy outcome (design #2). Upon commencement of breeding, one male was added to each cage of three females. Females were examined for vaginal plugs (evidence of copulation) the following morning at the beginning of the light cycle for a maximum of 4 days. The morning that the plug was found was considered day 0 of gestation and the females were then housed individually with nesting material. Pregnant C57BL/6 mice were administered 200 μl of PBS (vehicle), non-CpG ODN (300 μg/dam) or CpG ODN (3, 30, 300 μg/dam) by intraperitoneal injection (i.p.) on gestation day 6 at 9:00 a.m.

A total of 15 female mice per treatment group were mated for study design #1 and on the morning of gestation day 18, pregnant dams were sacrificed via CO_2 inhalation (number of gravid uteri per group was PBS, $n = 8$; non-CpG ODN, $n = 10$; 3 μg CpG ODN, $n = 8$; 30 μg CpG OND, $n = 9$; 300 μg CpG ODN, $n = 5$). Blood was collected from the abdominal aorta and serum stored at −80 °C for cytokine analysis. The gravid uteri were removed and weighed. Placentas and fetuses were removed from the uterine horns and preserved in 4% PBS-buffered paraformaldehyde, pH 7.4. Placental tissues were transected perpendicular to the long axis of the disc, paraffin-embedded, sectioned at 5 μm, and stained with hematoxylin and eosin for evaluation.

Pregnancy success rate, offspring survival and body weight gain were determined using a parallel experiment in which the dams were allowed to deliver and the offspring were examined for changes in immune function. A total of 35 females were mated per treatment group for study design

#2. Male and female offspring from three treatment groups: PBS-treated dams, non-CpG ODN, and 30 μg CpG ODN were evaluated for body weights at 3 weeks of age (126 pups from 21 litter) and 6 weeks of age (36 pups from 6 litter) (note: pups from the 3 μg CpG ODN group were not evaluated, and there were no viable pups at 3 weeks of age from the 300 μg CpG ODN group to evaluate).

2.3. Serum cytokine array

Serum was collected from dams on gestation day 18 and analyzed using a custom cytokine ELISA array (Searchlight™ Proteome Array, Pierce Endogen, Rockford, IL) according to the manufacturer's instructions. Antibodies specific for the cytokines IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p70, IFNγ, TNFα, and GM-CSF were spotted as a 3×3 grid pattern in each well of a 96-well ELISA plate. Detection limits ranged between 2.0 and 31.5 pg/ml. Standard ELISA procedures were employed followed by chemiluminescent imaging of the spots and data analysis using Array Vision™ (Imaging Research Inc., St. Catherines, ON). Cytokine concentrations were extrapolated from standard curves and the data expressed as pg/ml serum.

2.4. Statistical analysis

Data are expressed as arithmetic mean ± S.E.M. A one-way analysis of variance was conducted with a randomized complete block design for error control. Tukey's HSD test was used to establish significant differences in treatment groups for all continuous data. Categorical data (resorptions and malformations) were analyzed using Kruskal–Wallis one-way analysis of variance on ranks followed by Dunn's multiple comparison test. Dose–response trends were analyzed by linear regression where appropriate. Results were considered significantly different at $P < 0.05$.

3. Results

3.1. Pregnancy outcome, maternal and fetal weight and fetal resorptions

All data presented on fetal parameters, placental pathology and maternal cytokine levels was generated from dams sacrificed on day 18 of gestation (study design #1 in Section 2.2). All data on offspring and pregnancy outcome were generated using a parallel study in which natural birth was allowed to take place (study design #2 in Section 2.2). To determine pregnancy success and impact of treatment on litter size, a total of 35 mating pairs were established for each treatment group with 54% of the control dams having litters. Pregnancy success was markedly reduced (23%) in dams treated with the highest dose of CpG ODN (Table 1). Maternal treatment with 300 μg of CpG ODN during gestation also resulted in a dramatic decrease in litter size at birth. In fact, none of the offspring born to dams treated with 300 μg of CpG ODN survived more than a few days after birth (Table 1). In a parallel study in which dams were sacrificed at gestation day 18, dam weight ($R = -0.390$, $P < 0.037$) and gravid uterine weight ($R = -0.399$, $P < 0.032$) showed a modest but significant inverse dose–response relationship with increasing concentrations of CpG ODN (Fig. 1A and B). The average fetal weight at gestation day 18 was significantly lower in the 300 μg CpG ODN group (Fig. 1C). Amniotic fluid surrounding fetuses in the 300 μg CpG ODN group was pale red in color as opposed to clear in all other groups indicating possible intrauterine hemorrhage. In addition, all fetuses in the 300 μg CpG ODN group were pale white in color possibly due to poor blood supply.

The number of live fetuses per uterus on gestation day 18 (study design #1) was significantly reduced in the 300 μg CpG ODN group compared to the control group (Fig. 2A). Similarly, an increase in fetal resorptions was observed in the 300 μg CpG ODN group showing a 48% resorption rate (Fig. 2B). Low numbers of spontaneous fetal resorptions

Table 1
Pregnancy success and offspring survival following maternal treatment with CpG ODN on day 6 of gestation

Group	Pairing/litter (% success)	Litter size	Number of pups at birth	Number of pups at 3 weeks
PBS	35/19 (54)	6.7 ± 0.4[a]	126	112[b]
Non-CpG ODN				
300 μg/dam	35/27 (77)	7.1 ± 0.5	188	180[c]
CpG ODN				
3 μg/dam	35/19 (54)	6.5 ± 0.3	122	110[d]
30 μg/dam	35/22 (63)	6.5 ± 0.5	138	129[e]
300 μg/dam	35/8 (23)	1.1 ± 0.1[*]	9[*]	0[f,*]

[a] Mean ± S.E.M.
[b] Offspring loss due to cannibalism was 2 litter/14 pups.
[c] Offspring loss due to cannibalism was 1 litter/8 pups.
[d] Offspring loss due to cannibalism was 2 litters/12 pups.
[e] Offspring loss due to cannibalism was 1 litter/9 pups.
[f] Offspring loss due to cannibalism was 8 litters/9 pups.
[*] Significantly different from PBS group at $P < 0.01$.

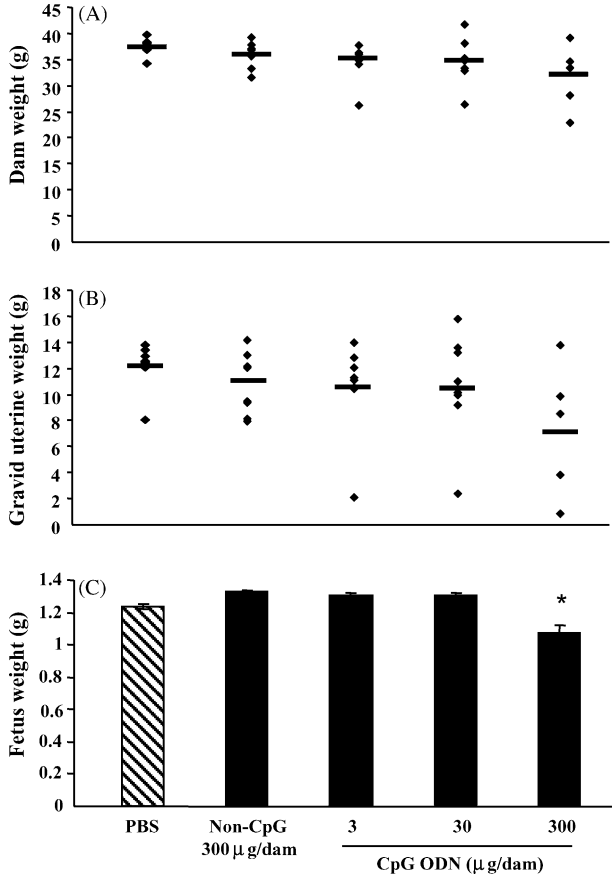

Fig. 1. Maternal and fetal body weight following maternal treatment with CpG ODN during gestation. C57BL/6 dams were treated with vehicle (PBS), non-CpG ODN (300 μg/dam) or CpG ODN (3, 30, or 300 μg/dam) in 200 μl PBS administered i.p. on day 6 of gestation. Maternal (A), gravid uterine (B) and fetal (C) weights were recorded on gestation day 18. Each point in (A and B) represents an individual dam or uterus weight, respectively (PBS, $n = 8$; non-CpG ODN, $n = 10$; 3 μg CpG ODN, $n = 8$; 30 μg CpG OND, $n = 9$; 300 μg CpG ODN, $n = 5$) and the group mean is indicated by the solid horizontal bar. Data in (C) is presented as mean ± S.E.M. (PBS, $n = 67$ fetuses from eight dams; non-CpG ODN, $n = 70$ fetuses from 10 dams; 3 μg CpG ODN, $n = 56$ fetuses from eight dams; 30 μg CpG OND, $n = 59$ fetuses from nine dams; 300 μg CpG ODN, $n = 23$ fetuses from four dams). *Significantly different from control group at $P < 0.05$.

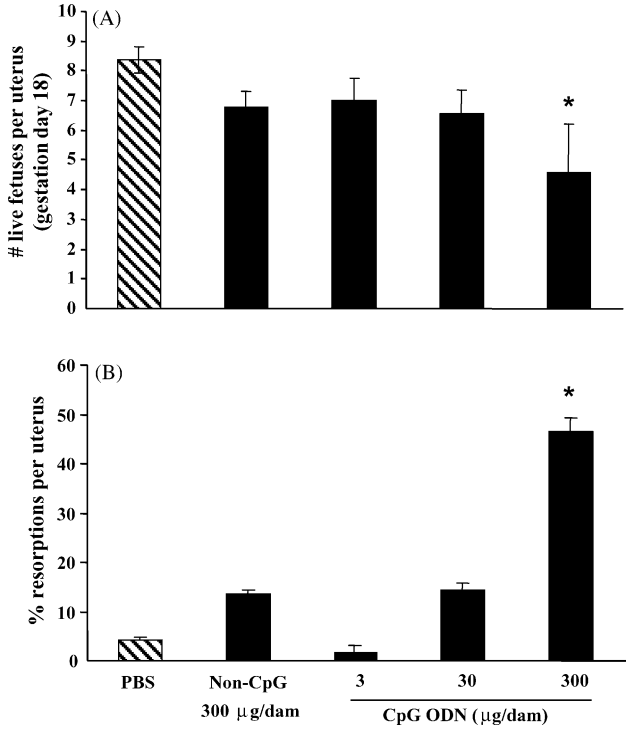

Fig. 2. Incidence of fetal resorptions following maternal treatment with CpG ODN during gestation. C57BL/6 dams were treated with vehicle (PBS), non-CpG ODN (300 μg/dam) or CpG ODN (3, 30, or 300 μg/dam) in 200 μl PBS administered i.p. on day 6 of gestation. The number of live fetuses per litter (A) and the percentage of fetal resorptions (B) was determined in gestation day 18 uteroplacental units ($n = 3/70$ fetal resorptions in 8 litter for controls, 11/81 resorptions in 10 litter from non-CpG group, 1/57 fetal resorptions in 8 litter from 3 μg CpG group, 10/69 fetal resorptions in 9 litters from 30 μg CpG group, and 20/43 fetal resorptions in 5 litter from 300 μg CpG group). *Significantly different from control group at $P < 0.05$.

are typically seen in C57BL/6 mice [23], and this was consistent in this study which showed 4.3% post-implantation fetal loss in the control group (Fig. 2B). Treatment-related effects on fetal death and resorption were not evident in dams treated with 300 μg of non-CpG ODN. Gender-matched pup weights from study design #2 in the PBS, non-CpG, and 30 μg CpG ODN groups were not different at 3 or 6 weeks of age (Table 2). Corresponding offspring weights were not available from the 300 μg CpG ODN group, due to the loss of pups shortly after birth.

3.2. CpG-induced placental pathology and fetal morphologic defects

Treatment-related changes in the incidence of craniofacial and limb defects resulted from high-dose CpG ODN exposure during gestation. Gestational exposure of pregnant

Table 2
Body weights 3 and 6 weeks after birth in neonates of mothers exposed to CpG ODN on gestation day 6

Sex	*n*	PBS	Non-CpG ODN (30 μg/dam)	CpG ODN (30 μg/dam)
3 weeks of age				
Male	21	9.2 ± 0.3[a]	9.5 ± 0.1	9.4 ± 0.2
Female	21	8.7 ± 0.2	8.7 ± 0.1	8.9 ± 0.2
6 weeks of age				
Male	6	18.7 ± 0.4	18.8 ± 0.4	19.4 ± 0.7
Female	6	17.7 ± 0.3	17.5 ± 0.4	17.2 ± 0.4

[a] Mean ± S.E.M.

(A)	PBS	non-CpG	3 μg CpG	30 mg CpG	300 mg CpG
Malformation incidence	2/67 (3%)	0/70 (0%)	2/56 (4%)	0/59 (0%)	11/23 (48%)*

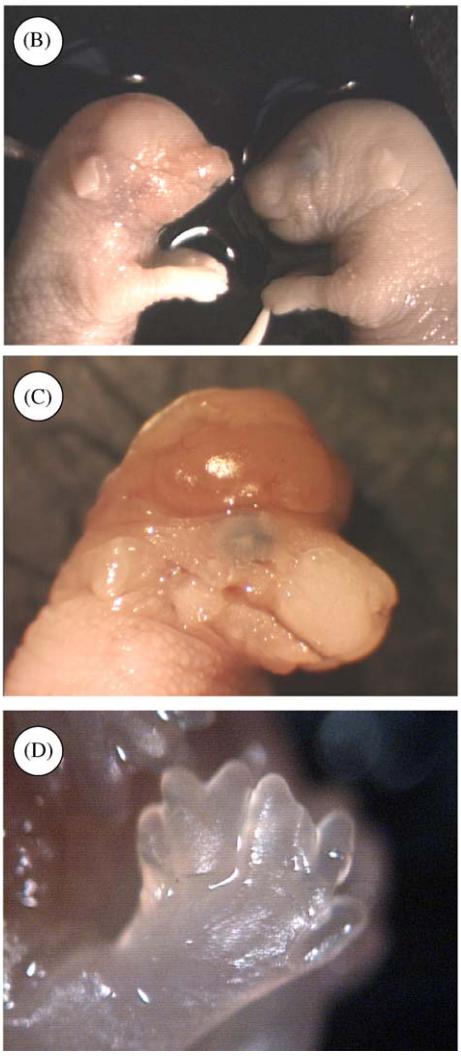

Fig. 3. Incidence of fetal craniofacial and distal limb malformation following maternal treatment with CpG ODN during gestation. C57BL/6 dams were treated with vehicle (PBS), non-CpG ODN (300 μg/dam) or CpG ODN (3, 30, or 300 μg/dam) in 200 μl PBS administered i.p. on day 6 of gestation. Fetuses were removed on gestation day 18 and examined for external maldevelopment. Malformation incidence (Table A) is expressed as number of fetuses with one or more malformations/total fetuses examined (% malformed). Fetal malformations included anophthalmia and mandibular brachygnathia (Panel B, left is a fetus from 300 μg CpG ODN group and right is a fetus from control group), exencephaly (Panel C), and syndactyly/polydactyly (Panel D). *Significantly different from control group at $P < 0.05$.

dams to 300 μg of CpG ODN, in addition to dramatically increasing fetal loss and resorption, resulted in 48% (11 of 23 pups, Fig. 3A) of viable fetuses showing craniofacial and/or distal limb defects on gestation day 18. The most common defects associated with this dose of CpG ODN included anophthalmia (Fig. 3B), mandibular brachygnathia (Fig. 3B), exencephaly (Fig. 3C), syndactyly/polydactyly, and under-developed digits (Fig. 3D). There were no visible fetal craniofacial or distal limb defects observed following exposure of dams to non-CpG ODN or 30 μg of CpG ODN. Only two fetuses from the 3 μg CpG ODN group had malformations, one displaying craniofacial shortening and the other oligodactyly. As with resorptions, a low-level rate of spontaneous defects occurred in the control group (3%, 2 of 67 pups, Fig. 3A), including one syndactyly, and one microcephaly with microopthalmia. This is consistent with the background incidence of eye malformations that has been reported to occur in inbred C57BL/6 mice [24].

Fetal malformation may occur subsequent to inflammation-induced placental damage [25]. As such, gestation day 18 placentas were examined histologically following hematoxylin and eosin staining. Longitudinal transection perpendicular to the long axis of the disc allowed observation of the following layers from maternal to fetal side: myometrium, trophoblast giant cells and syncitiotrophoblasts, glycogen-rich trophoblasts, and the highly vascular labyrinth layer. Placentas from control dams displayed rare small focal areas of necrosis that predominantly targeted endothelium in the labyrinthine layer, with karyolysis and pyknosis, cellular fragmentation, hypereosinophilic cytoplasm, collapse of vascular patency, and occasional areas of dystrophic mineralization (Fig. 4A). Scattered necrotic trophoblasts were also seen in the syncitial layer, and rarely in the trophoblast giant cell layer. These rare lesions were visualized in the non-CpG ODN, 3 μg CpG ODN and 30 μg CpG ODN placental samples, although the most dramatic pathology was observed following treatment with 300 μg of CpG ODN. In the high dose group, there was a profound loss of trophoblasts in the giant cell layer, the syncitial layer, and the glycogen trophoblast clusters, with marked vascular damage, necrosis, calcification and accompanying suppurative inflammation in the labyrinthine layer (Fig. 4B and C), pathology suggestive of compromised circulation to the fetus.

ELISA arrays were conducted to determine whether the morphologic alterations observed in dams treated with the 300 μg dose of CpG ODN were accompanied by elevated inflammatory or T_H1 cytokines. Maternal serum collected on gestation day 18 showed increased levels of the T_H1 cytokines, IL-2, IL-10, and IL-12p70 (Fig. 5). Maternal serum concentrations of TNFα were slightly elevated in the 300 μg CpG group relative to the control group (Student's *t*-test $P < 0.05$), while the levels of GM-CSF, IL-1β, IL-6, IFNγ (Fig. 5) and IL-4 (data not shown) were not affected by any treatment.

4. Discussion

Diverse vaccination procedures in potentially pregnant women are becoming increasingly common and are considered to be associated with relatively low risk based on several recent studies using tetanus toxoid, *Haemophilus influenzae* type B, varicella, influenza, and respiratory syncitial virus vaccines [26–30]. However, limited preclinical data have

been published regarding the reproductive toxicity of preventive vaccines or vaccine formulations for infectious disease indications. Specifically, attempts to document potential causal relationships between gestational vaccine administration and birth defects have not been a high priority. This, in part, is because single administration of vaccines or medicinal agents in women of good health and of childbearing age has historically been considered a low-risk procedure. As such, reproductive toxicology studies are not usually required for the registration of such agents that are intended for single administration [31], and the potential benefits of maternal vaccination to protect the unborn fetus are generally presumed to outweigh the risks of harm to the developing baby. Our results indicate that even a single administration of CpG ODN during pregnancy can have detrimental effects on pregnancy outcome in a murine model. Importantly, our data show abortion and maldevelopment at a maternal dose of 15 mg/kg CpG ODN. The highest dose reported in published human clinical trials is 0.5 mg/kg [32], which is approximately 30-fold lower than the dose causing malformations in the present study. Nevertheless, these findings underscore the need for detailed toxicological studies including reproductive and developmental toxicology, since the differential sensitivity between rodents and humans is poorly understood at the present time.

Increased fetal toxicity and maldevelopment were only observed following treatment of dams with the highest dose of CpG ODN, indicating the lack of a dose–response relationship. In contrast to the present findings, in vitro studies have demonstrated that the production of inflammatory cytokines increases with increasing concentration of CpG ODN in human [33] and murine [33,34] immune cells. The lack of dose–response in the present study may be a result of the complex interaction between CpG-induced inflammation, placental damage and fetal toxicity. For example, the peak level and duration of elevation of inflammatory cytokines may have a profound impact on homeostasis of the placental-fetal unit. Although peak levels of serum cytokines were not established in the present study, the results demonstrate that persistent cytokine elevation, at least to gestation day 18, was observed in dams treated with 300 μg of CpG ODN, an effect not observed in any other treatment group. This suggests that the duration of the inflammatory response to CpG ODN may be important in the induction of fetal toxicity and malformation. Using a different strain of mice, Ito et al. [35] did not report any maternal or fetal toxicity or maldevelopment following exposure to 150 μg of CpG ODN on gestation day 10. This lack of effect may be related to timing of exposure since

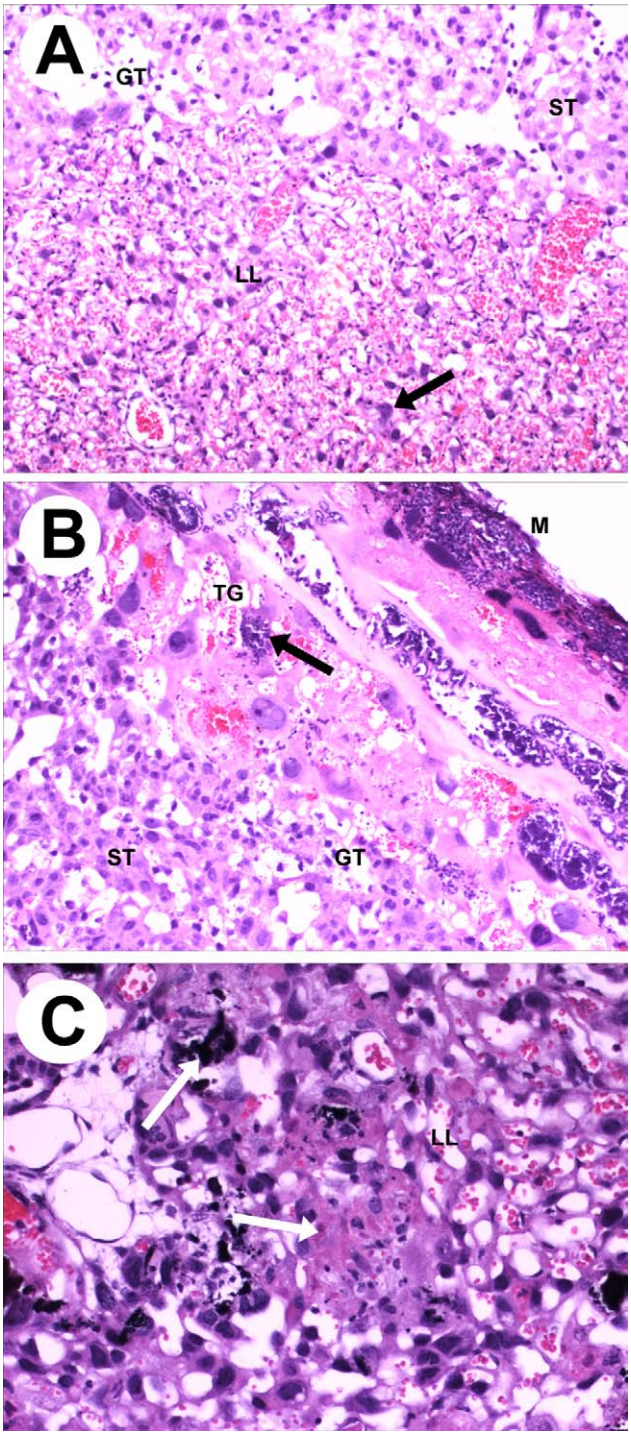

Fig. 4. Hematoxylin and eosin staining of the placenta following maternal treatment with CpG ODN during gestation. C57BL/6 dams were treated with vehicle (PBS), non-CpG ODN (300 μg/dam) or CpG ODN (3, 30, or 300 μg/dam) in 200 μl PBS administered i.p. on day 6 of gestation. Placentas (PBS, $n = 56$; non-CpG ODN, $n = 51$; 3 μg CpG ODN, $n = 51$; 30 μg CpG OND, $n = 48$; 300 μg CpG ODN, $n = 22$) were removed from the uterus and fixed in 4% buffered paraformaldehyde. Placental tissues for histopathological analysis were transected perpendicular to the long axis of the disc and evaluated for evidence of inflammation, necrosis and mineral deposition. The black arrows in panels A and B represent a necrotic endothelial cell in the labrynthine layer and a necrotic giant trophoblast, respectively. The white arrows in panel C identify clusters of necrotic endothelial cells in labyrinthine layer, with loss of vascular patency. Panels A, B and C show representative micrographs of a placental discs from the control group (100×), 300 μg CpG ODN group (100×) and 300 μg CpG ODN group (200×), respectively. M: myometrium; TG: trophoblast giant cells; ST: syncitiotrophoblasts; GT: glycogen-rich trophoblasts; LL: labyrinthine layer.

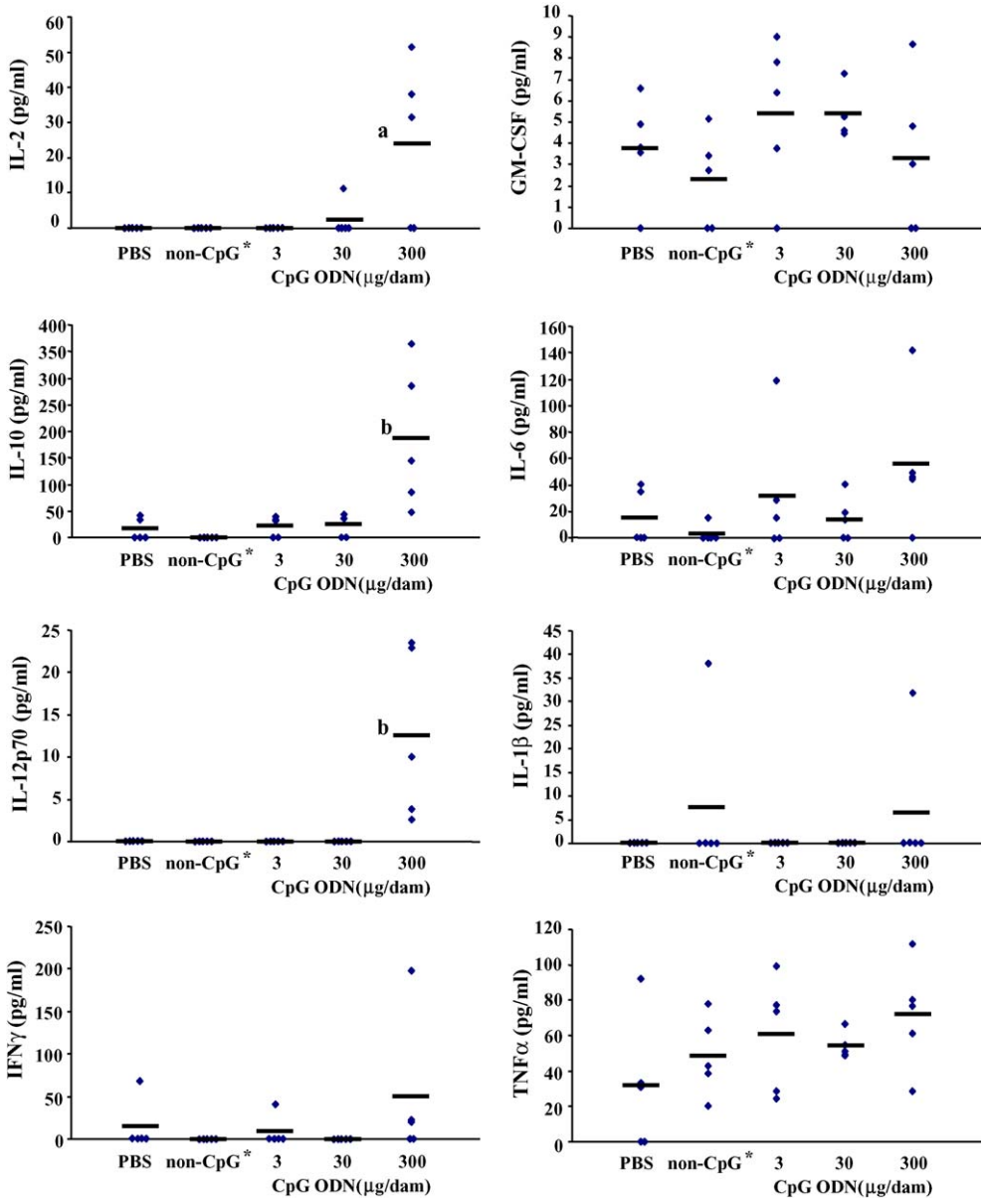

Fig. 5. Cytokine levels in the serum of dams treated with CpG ODN. C57BL/6 dams were treated with vehicle (PBS), non-CpG ODN (300 μg/dam) or CpG ODN (3, 30, or 300 μg/dam) in 200 μl PBS administered i.p. on day 6 of gestation. Dams were euthanized on day 18 of gestation, serum obtained and cytokine levels determined by an ELISA array (see Section 2). Each diamond represents cytokine levels from a single dam and the mean for each group ($n = 5$) is represented by black bars. *Non-CpG ODN was administered at a dose of 300 μg/dam corresponding to the highest dose of CpG ODN used. Significantly different from all other groups at $^{a}P < 0.05$; $^{b}P < 0.005$.

the majority of placentation occurs prior to gestation day 10. Alternatively, BALB/c mice may be less sensitive to CpG ODN due to lower TLR9 expression relative to C57BL/6 mice [36]. Additional studies investigating the time line of cytokine production and its association with damage to the placenta and fetal toxicity will be important for accurate risk assessment.

The normal maternal immune system shifts to a predominantly T_H2 cytokine profile in order to help support pregnancy, e.g., placental growth and function [25] and to minimize rejection responses toward fetal tissue during pregnancy [37,38]. In rodent models, vaccination procedures that would be predicted to cause T_H1 shifts have been associated with increased birth defects such as developmental neurotoxicity following pertussis vaccination [39], congenital hydrancephaly and porencephaly caused by bluetongue vaccine [40], and ureteral stenosis and triphalangeal hallux following exposure to yellow fever vaccine early in pregnancy [41]. We observed that maternal exposure to a high dose of CpG ODN caused a pronounced shift toward a T_H1 profile in lieu of a predominant T_H2 profile. This T_H1 shift was characterized by increases in IL-2, IL-10 and IL-12p70. While these studies were not designed to determine whether this was a direct cause–effect relationship between the T_H1

shift and fetal loss/maldevelopment, the findings are consistent with previous studies in which spontaneous resorption in mice was attributed to activation of NK cells, macrophages, and increased liberation of T_H1-type cytokines including, IL-2, IL-12 and IFNγ, as well as the pro-inflammatory cytokine TNFα [42–44]. In a similar light, neutralization of TNF and IL-1 receptors prevented stress-induced abortions in mice [45].

Extensive pathological damage to the placenta including inflammation and vascular collapse was evident in dams exposed to the high dose of CpG ODN. It is possible that the inflammatory changes observed in the placenta lead to altered blood flow as well as nutrient and waste distribution to and from the fetus. A recent human study demonstrated that utero-placental pathology, including placental inflammation, vascular pathology, vascular infarction and villous dysmaturity, was a leading cause of intrauterine fetal death [46]. Therefore, it is likely that placental pathology contributed to the unexpected 48% resorption rate and increased incidence of fetal malformation that occurred in these mice.

In summary, the present data provide additional evidence suggesting that induction of a T_H1 cytokines during pregnancy increases the risk of abortion and morphologic defects in mice. Understanding the apparent lack of dose–response for CpG ODN will be critical to characterizing this risk in humans. In addition, the differential sensitivity of humans and mice to CpG motifs represents a significant data gap that warrants further research. The relative contribution of individual T_H1 cytokines or cytokine combinations to this outcome remains uncertain at best, but represents a critical aspect of vaccine development that has received limited attention. The present data support the need for further investigation of teratogenesis that may occur secondary to vaccination-induced T_H1 cytokine profile shifts in pregnancy.

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