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TLR9 Provokes Inflammation in Response to Fetal DNA: Mechanism for Fetal Loss in Preterm Birth and Preeclampsia


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Preterm birth, the major cause of neonatal mortality in developed countries, is associated with intrauterine infections and inflammation, although the exact mechanisms underlying this event are unclear. In this study, we show that circulating fetal DNA, which is elevated in pregnancies complicated by preterm labor or preeclampsia, triggers an inflammatory reaction that results in spontaneous preterm birth. Fetal DNA activates NF-κB, shown by IkBα degradation in human PBMCs resulting in production of proinflammatory IL-6. We show that fetal resorption and preterm birth are rapidly induced in mice after i.p. injection of CpG or fetal DNA (300 μg/dam) on gestational day 10–14. In contrast, TLR9−/− mice were protected from these effects. Furthermore, this effect was blocked by oral administration of the TLR9 inhibitor chloroquine. Our data therefore provide a novel mechanism for preterm birth and preeclampsia, highlighting TLR9 as a potential therapeutic target for these common disorders of pregnancy. The Journal of Immunology, 2012, 188: 000–000.

Abbreviations used in this article: CLQ, chloroquine; DMEM–FCS–PS, DMEM–FCS–proliferation serum; FACS, fluorescence-activated cell sorter; PTB, preterm birth; SCT, spongiform cytotrophoblast; UM, uterine muscle.

Footnotes:
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A.S.-N. and S.C.C. conceived ideas, designed and performed the experiments of Figs. 1–4, and analyzed data. S.C.C. wrote the manuscript. S.B.C. provided assistance with the TLR9-deficient in vivo model, which was performed in the laboratory of K.A.F., who provided the TLR9-deficient mice. C.M. and L.K. performed immunohistochemistry. B.D. performed histological analyses. S.D. and J.J.O. helped with histological analyses and conceived ideas. L.A.J.O. conceived ideas, edited the manuscript, and funded and directed the project.

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Abbreviations used in this article: CLQ, chloroquine; DMEM–FCS–PS, DMEM–glutamax supplemented with 10% FCS and 1% penicillin–streptomycin; GCT, giant cell trophoblast; ODN, oligonucleotide; PEC, preeclampsia; PMN, polymorphonuclear cell; PTB, preterm birth; SCT, spongiform cytotrophoblast; UM, uterine muscle.

Preterm birth, defined as delivery before 37 wk gestation, has become an epidemic in developed countries. Indeed, PTB and preeclampsia (PEC) are leading causes of maternal and neonatal death worldwide. Premature infants who survive are inflicted with many lifelong handicaps including cerebral palsy and mental retardation (1). The most significant risk factor for PTB is a prior preterm delivery, although both PTB and PEC are associated with multiple other factors including infection and inflammation (2). Indeed, the innate immune system has been shown to play a role during pregnancy and immunogenic disorders of pregnancy. TLRs are an important class of pathogen recognition receptors, which signal during infection to bring about immune reactions that have been implicated in PTB (3). TLR2 and TLR4, which signal in response to bacterial lipoproteins and LPS, respectively, are highly expressed in the amniotic tissues of women who delivered prematurely, with increased expression of TLR2 and TLR4 in the chorioamniotic membranes (4–8). The Asp299Gly polymorphism, a variant of the human TLR4 gene, has been associated with PTB (8). However, despite these associations, it remains unclear what exact role microbial infection has in PEC as microbial products are rarely detected in the maternal circulation, and furthermore it has been estimated that only 30% of PTB cases demonstrate evidence of microbial infection (9). To further this discrepancy, antibiotic treatment has never been shown to decrease rates of preterm delivery (10). In one study, antibiotic treatment of at-risk patients caused delivery earlier than that for patients not treated with antibiotics, highlighting a lack of knowledge of the exact mechanism underlying PTB and PEC (11–13). Although bacterial and viral factors may incite the inflammatory process without evidence of infection by cultures or histology, it is possible that another more sterile process might be at work. Notably, samples of amniotic fluid and placenta were examined for bacterial products at elective section of term pregnancies, and 70% had evidence of the presence of microbial products by fluorescence in situ hybridization without infection or symptoms of labor or PEC (14). This presents an incongruity with respect to factors from an infective source being the sole cause of inflammatory disorders of pregnancy even at a local level. The concept that, as fetal cells die from whatever insult (subclinical infection, growth restriction, trauma), they release hypomethylated fetal DNA fragments that function as a “danger signal” from the fetus to the mother in order to start an inflammatory process to begin labor would be a unifying mechanism for many of the risk factors for PTB.

A possible clue as to the cause of PTB and PEC is the observation that cell-free fetal DNA can be found in maternal plasma, and furthermore circulating levels of free fetal DNA are significantly elevated in complicated pregnancies (15). This source of free fetal
DNA is likely to arise from either destruction by the maternal immune system of fetal cells, which occasionally cross the placental barrier, or from placental and fetal cells that undergo apoptosis (16). Although normal pregnancies have a certain amount of fetal DNA present within the maternal circulation, this is quickly cleared after delivery (17). However, many different complications of pregnancy are associated with an increased amount of fetal DNA being found in the maternal circulation, with PEC and PTB having free fetal DNA concentrations between 2 and 10 times higher, respectively, than those of normal controls at the same gestational age (18–20). As a result, detection and quantification of free fetal DNA is being developed as an early diagnosis for these disorders (4, 19).

Fetal DNA is hypomethylated, and because TLR9 senses hypomethylated DNA normally found in abundance in microbial DNA, we hypothesized that it might sense fetal DNA and provoke an inflammatory reaction possibly leading to PTB (21). In this study, we report that human fetal DNA can indeed activate TLR9 in vitro. We also demonstrate in vivo that when injected into pregnant mice, fetal DNA leads to an inflammatory response that results in fetal loss and poor outcome during pregnancy that is TLR9 dependent. Furthermore, using this model we show that this effect can be blocked after oral treatment of pregnant dams with the TLR9 inhibitor chloroquine, highlighting a potential clinical application. Our work therefore provides a novel mechanism for fetal loss in PTB and PEC and suggests that targeting of TLR9 could have clinical utility.

Materials and Methods

**Cell culture**

PBMCs isolated from female donors (both nonpregnant and pregnant) and Namalwa cells were cultured in DMEM-glutamax (Life Technologies) supplemented with 10% FCS (Sigma) and 1% penicillin–streptomycin (Sigma) (DMEM–FCS–PS) and seeded at 2.5 × 10^6 cells/ml for experimental assays. Primary bone marrow-derived macrophages (BMDMs) were generated from bone marrow isolated from wild-type mice and cultured in DMEM–FCS–PS supplemented with M-CSF (20%) L929 cell supernatant) for 10 d and seeded at 5 × 10^5 cells/ml for assay.

**iκBα degradation**

Cells were stimulated for various times with either fetal DNA (22-wk female fetus; Biochain), adult DNA (blood of a 50-y-old female; Biochain) or human CpG (Invivogen) at 1.5 μg/ml or 3 μg/ml. iκBα degradation was measured by immunoblot analysis using an anti-iκBα Ab (gift from Prof. R. Hay, University of Dundee, Dundee, U.K.). The mAb used to detect β-actin was from Sigma.

**ELISA analysis**

After stimulation of cells for 18 h, human and murine IL-6 were measured in supernatants as per the manufacturer’s protocol (R&D Systems).

**Inhibition of TLR9**

Namalwa cells and PBMCs isolated from pregnant donors were pretreated with either chloroquine (50 μM; Sigma) for 1 h or a synthetic inhibitory oligonucleotide (ODN) (12 μg/ml, ODN TTAGGG; Invivogen) for 6 h prior to stimulation with fetal DNA (1.5 μg/ml).

**Restriction enzyme assay**

One microgram per milliliter of fetal DNA or adult DNA was incubated for 24 h at 37 °C alone or with HPA II (New England Biolabs) or S-adenosyl methionine CpG methyltransferase enzyme and visualized by gel electrophoresis.

**Animals and treatment**

Male and female specific pathogen-free BALB/c mice (obtained from Harlan UK) or C57Bl/6 and C57Bl/6 TLR9-ko mice (a gift from K. Fitzgerald, University of Massachusetts) were obtained at 6–12 wk of age. Males were housed individually, and females were housed in groups of five per cage. Food and water were supplied ad libitum. Upon commencement of breeding, one male was added per two females, and females were examined for presence of vaginal plugs (evidence of copulation) the following morning. The morning that the plug was found was considered day 1 of gestation. Pregnant mice were administered PBS (vehicle), LPS (300 μg/dam), CpG ODN (300 μg/dam), fetal DNA (300 μg/dam), or adult DNA (500 μg/dam) by i.p. between gestation days 10 and 14. To determine if chloroquine could be used to prevent fetus resorption, chloroquine (CLQ; 1 mg/dam) was administered orally at 3 h prior to injection with fetal DNA followed by an additional 2 mg/dam CLQ administered orally 3 h after injection with fetal DNA. Forty-eight hours after injection of fetal DNA, pregnant dams were sacrificed via CO2 inhalation, terminal blood was collected, and serum stored at −80 °C for cytokine analysis. The gravid uteri were removed and weighed and the number of resorbed fetuses determined. Placentas and fetuses were preserved in 10% formalin until histological analysis. All animal procedures were approved by the Ethical Review Board, Trinity College Dublin, in accordance with the Cruelty to Animals Act 1876.

**Immunohistochemistry**

Mouse gestational sacs were removed from uterine horns and placed in 10% buffered formalin and paraffin embedded 24 h after fixation. Samples were stained with H&E. IL-6 and TNF-α were detected using a rabbit-specific HRP/3,3′-diaminobenzidine detection immunohistochemistry kit (Abcam).

**Statistical analysis**

For in vitro studies, Student t test was performed to test statistical significance at p < 0.05; data are expressed as means ± SD. For animal studies, one-way ANOVA analysis was performed to test statistical significance at p < 0.05.

**Results**

Hypomethylated fetal DNA triggers inflammation in human B cells and PBMCs

Initially, we tested a model cell line, Namalwa, a human B cell line that expresses high levels of TLR9. Fetal DNA activated the proinflammatory transcription factor NF-κB as evidenced by iκBα degradation at 5 min stimulation (Fig. 1A, top panel), whereas CpG DNA was active at 15 min (Fig. 1A, middle panel). Adult DNA, which is not hypomethylated, had no effect (Fig. 1A, bottom panel). We next tested the effect of fetal DNA in a more relevant cell population, PBMCs prepared from female donors (who were not pregnant but were of child-bearing age). Both fetal DNA and CpG induced iκBα degradation within 5 min of stimulation (Fig. 1B, top and middle panels, respectively). Adult DNA did not induce iκBα degradation (Fig. 1B, bottom panel). Both fetal DNA and CpG induced IL-6 production in PBMCs up to 4-fold over control (Fig. 1C). Furthermore, fetal DNA significantly induced IL-6 production in PBMCs isolated from pregnant women (n = 5, average donor age 32.6 y, average gestational age 25 wk, two donors had no prior pregnancy, three donors had one prior pregnancy) (Fig. 1D). Fetal DNA did not, however, induce IL-6 in primary murine BMDMs, despite CpG being capable of inducing an IL-6 response in this cell type (Fig. 1E). LPS, which was included as a control, also induced IL-6 in these cells (Fig. 1E). We next analyzed the methylation status of fetal DNA by performing a restriction digest with HPAII, an enzyme that cleaves DNA at unmethylated CpG islands. Fetal DNA was cleaved by HPAII (Fig. 1F, compare lane 2 with lane 1), and this cleavage was prevented by pretreatment with CpG methyltransferase and S-adenosyl methionine, which adds methyl groups to CpG motifs, prior to HPAII digestion (Fig. 1F, lane 3). Adult DNA, which is hypermethylated, remained uncleaved after HPAII digestion (Fig. 1F, lane 5).

**Fetal DNA induces fetal resorption in mice**

We next tested the effect of fetal DNA in pregnant mice. In pregnant mice, previous studies have shown that i.p. adminis-
degradation in Namalwa cells stimulated with fetal DNA, CpG, and adult DNA. (B) IκBα degradation in PBMCs stimulated with fetal DNA, adult DNA, and CpG DNA. (A and B) are representative of three independent experiments. (C) ELISA analysis of IL-6 response in PBMCs from female donors who were not pregnant, stimulated with fetal DNA and CpG. Values are means + SD, n = 3, *p < 0.05 (representative of three non-pregnant female donors). (D) ELISA analysis of IL-6 response to fetal DNA in PBMCs from pregnant donors. Values are means + SD, n = 3, *p < 0.05 (representative of five pregnant donors). (E) ELISA analysis of IL-6 response in primary BMDMs stimulated with fetal DNA, CpG, and LPS. Values are means + SD, n = 3, *p < 0.05 (representative of three independent experiments). (F) Fetal DNA contains hypomethylated CpG as shown by HPAII restriction enzyme digestion of nonmethylated DNA (lane 3) compared with fetal DNA pretreated with CpG methyltransferase and S-adenosyl methionine (lane 4) and adult DNA (lane 6) for 24 h. Control fetal DNA (lane 2) and adult DNA (lane 5) were not exposed to the restriction enzyme. Representative of three independent experiments.

Fetal DNA triggers inflammation in Namalwa B cells and human PBMCs. (A) IκBα degradation in Namalwa cells stimulated with fetal DNA, CpG, and adult DNA. In contrast, the placental sites of mice injected with PBS or adult DNA were without significant infiltrates and remained intact (Fig 2B, micrographs 1 and 2, respectively). In micrographs 1 and 2, GCTs of the placenta are attached to UM, and occasional PMNs are seen within the maternal blood vessel in a singular arrangement (25). Notably, whereas CpG induced systemic production of a range of cytokines (IFN-γ, IL-12p70, mKC, TNF-α, IL-10) in mice, fetal DNA did not induce this response suggesting a localized effect (data not shown). To confirm whether this was indeed a localized effect, immunohistochemistry was performed on tissue sections for the presence of TNF-α (Fig 2C) and IL-6 (Fig 2D), two cytokines that have previously been implicated in inflammatory disorders of pregnancy (26). Dams treated with PBS and adult DNA demonstrated no evidence of TNF-α in the endometrium (Fig 2C, micrographs 1 and 2, respectively), whereas dams administered CpG and fetal DNA showed positive immunohistological staining for TNF-α (Fig 2C, micrographs 3 and 4, respectively). Similarly, there was a lack of IL-6 staining in dams administered PBS and adult DNA (Fig 2D, micrographs 1 and 2, respectively) compared with dams administered CpG DNA (Fig 2D, micrograph 3) and fetal DNA (Fig 2D, micrograph 4). Infiltration of inflammatory cells was observed in sections from mice administered CpG and fetal DNA (Fig 2E, first column, middle and bottom panels). Colocalization of PMNs and monocytes with the inflammatory cytokines is indicated by circles (Fig 2E, middle and right-hand columns, middle and bottom panels).
**Fetal DNA is sensed by TLR9 to induce fetal resorption**

Having already demonstrated that fetal DNA could activate NF-κB and induce IL-6 in vitro and also induce fetal loss and cytotrophoblastic inflammation in vivo in mice, we next tested whether its effect was TLR9 dependent. As we had previously seen that hypomethylated fetal DNA activated Namalwa cells, which express high levels of TLR9, we sought to determine whether fetal DNA could be sensed by TLR9. Thus, using our model of PTB, we compared fetal resorption in wild-type C57bl/6 and C57bl/6 TLR9-deficient mice (Fig. 3). Fetal DNA induced only 12% ($n=3$ of 25) resorption in TLR9-deficient mice compared with 85% ($n=29$ of 34) resorption in wild-type mice (Fig. 3A). The resulting fetal resorption correlated with a significant reduction in fetal weight in wild-type mice (Fig. 3B). H&E staining of the placenta after maternal treatment during gestation with adult DNA (top row), CpG (middle row), or fetal DNA (bottom row) demonstrated the presence of TNF-α or IL-6 protein within the cytoplasm of neutrophils and monocytes (25). Original magnification $\times$400.

**CLQ prevents fetal DNA-induced fetal loss in mice**

Next, we investigated the potential therapeutic effect of the TLR9 inhibitor CLQ in our model. CLQ has been shown to inhibit TLR9 by blocking acidification of the endosome, which is required for TLR9 signaling (27). First, we observed that pretreatment of Namalwa cells with CLQ (Fig. 4A) or an inhibitory ODN to TLR9 (Fig. 4B) for 1 h or 6 h, respectively, prior to stimulation with fetal DNA (1.5 μg/ml) prevented fetal DNA-induced IkBα degradation in these cells compared with untreated cells stimulated with fetal DNA (compare bottom panel with top panel in...
FIGURE 3. Fetal DNA is sensed by TLR9 to induce fetal resorption. (A) TLR9-deficient mice are resistant to fetal DNA as shown by measurement of fetal resorption in pregnant C57bl/6 wild-type and C57bl/6 TLR9-deficient mice administered i.p. fetal DNA on gestation day 10–14 and sacrificed 48 h later. n indicates number of resorbed fetuses out of total number of fetuses. *p < 0.05 (significantly different from control group). (B) TLR9-deficient mice are resistant to resorption induced by fetal DNA as shown by fetus weights. (C) H&E staining of the placenta from C57bl/6 wild-type and TLR9-deficient mice after maternal treatment during gestation with fetal DNA (1 and 2). Arrow indicates calcification. Sections were stained for the presence of TNF (3 and 4) or IL-6 (5 and 6), indicated by presence of brown staining. Original magnification ×400. (D) Representative uterine horns harvested on gestational day 14 from C57bl/6 wild-type and TLR9-deficient mice treated on gestational day 12 with fetal DNA demonstrating fetal DNA-induced fetal resorption is TLR9 dependent.

Discussion

Despite advances in the management of high-risk pregnancies, such as those complicated by PTB and PEC, the incidence of these complications remains high, with knowledge of the underlying mechanisms still unclear. However, it has become obvious that there is an intimate relationship between the maternal immune system, the fetus, and their relative environments, and recent studies have begun to investigate these relationships. In this study, we have analyzed the proinflammatory potential of human fetal DNA, which is found at increased concentrations in the circulation of pregnant women undergoing PTB or PEC. Fetal DNA caused activation of the transcription factor NF-κB, leading to the production of proinflammatory IL-6. Furthermore, using a mouse model, we investigated the consequences of fetal DNA administration in pregnant BALB/c and C57bl/6 mice. We observed that when injected i.p. on day 10–14 of gestation, fetal DNA caused rapid fetal resorption in both BALB/c and C57bl/6 mice and a localized inflammatory response in the uterus, as shown by infiltration of PMNs and monocyes and cytokine production by these cells. This inflammatory effect of fetal DNA is dependent on TLR9, as C57bl/6 TLR9-deficient mice experienced significantly reduced fetal resorption in response to fetal DNA. Furthermore, oral administration of the TLR9 inhibitor CLQ significantly protected BALB/c mice from fetal resorption. Our data provide a new model for PTB, a recently reported deficiency in this field, highlighted by a call by Nature Medicine for better models to study this clinical dilemma, with hopes that research resulting from these models would lead to an increased understanding of PTB, ultimately leading to a decreased incidence and improved neonatal outcome (28). Our study lends itself to this cause. CLQ has been given to pregnant mothers with malaria and systemic lupus erythematosus for decades. CLQ crosses the placenta as its metabolites are found in the cord blood, systemic blood, and urine of neonates delivered from mothers treated with CLQ (29). As TLR9 responsive cells are maternal monocytes, dendritic cells, NK cells, macrophages, and trophoblasts of the human placenta, it is likely that such a therapy would reach the local cells likely to be involved. Our observations highlight a further therapeutic use for CLQ during pregnancy.

In our study, we observed infiltration of leukocytes into the murine placenta after administration of fetal DNA. This inflammatory cell infiltration was notably absent in mice administered...
either PBS or adult DNA and in TLR9-deficient and CLQ-treated mice. This inflammatory infiltration correlates with observations in women of increased infiltration of neutrophils into the uterine muscle after the onset of labor (30). Furthermore, we observed localized staining of proinflammatory IL-6 and TNF-α in placental tissues from mice administered fetal DNA. Again, this was absent in mice administered either PBS or adult DNA and in TLR9-deficient and CLQ-treated mice. IL-6 has previously been associated with PTB, and the concentration of IL-6 present in amniotic fluid is a sensitive test for antenatal diagnosis and can predict perinatal morbidity (31, 32). Absence of a proinflammatory profile in the systemic circulation of mice administered fetal DNA suggests a localized effect. This is further supported by a lack of activation of BMDMs while fetal DNA did activate both a Namalwa B cell line and PBMCs from both pregnant and nonpregnant donors, again suggesting that the target cell is localized to the uterus, perhaps being either a monocyte or NK cells. Indeed, TLR9 is expressed by numerous cells of the immune system including NK cells and monocytes, with its highest expression on dendritic cells and B lymphocytes, and reduced expression on monocyte-derived macrophages (33–39). Furthermore, why BMDMs did not respond to fetal DNA but did respond to CpG DNA is not clear but could be due to macrophages being less responsive overall to TLR9 stimulation.

Increased concentrations of fetal DNA present in the maternal circulation is therefore a likely trigger for inflammation that results in complications of pregnancy including PTB, PEC, growth retardation, and stillbirth. High levels of free fetal DNA at 11–14 wk gestation are predictive of poor fetal outcome including delivery before 35 wk gestation and increased likelihood of growth restriction or PEC (40). Pregnant women who experience one of the most severe forms of PEC, HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count) have the highest levels of cell-free fetal DNA in the maternal circulation, nearly 10-fold higher than those in normal pregnancies at the same gestational age (41). High levels of fetal DNA are also detected in the maternal circulation during placental abruption, which results in delivery, disseminated intravascular coagulation, and often fetal death (42). It is likely that high levels of free fetal DNA in maternal circulation functions as a danger signal to the mother that fetal cells are dying, leading to inflammation and delivery or death of the fetus. One must also consider that genetic variation in TLR9 may predispose women to fetal loss. We are currently examining this possibility.

Our observation raises the possibility that inhibition of TLR9 through administration of CLQ or ODNs that antagonize TLR9 to mothers with high levels of fetal DNA detected in their circulation might prevent two of the most common and detrimental disorders of pregnancy.
Disclosures
The authors have no financial conflicts of interest.

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