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Increased TLR4 expression in murine placentas after oral infection with periodontal pathogens

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Abstract

Maternal periodontitis has emerged as a putative risk factor for preterm births in humans. The periodontitis-associated dental biofilm is thought to serve as an important source of oral bacteria and related virulence factors that hematogenously disseminate and affect the fetoplacental unit; however the underlying biological mechanisms are yet to be fully elucidated. This study hypothesized that an oral infection with the human periodontal pathogens *Campylobacter rectus* and *Porphyromonas gingivalis* is able to induce fetal growth restriction, placental inflammation and enhance *Toll-like* receptors type 4 (TLR4) expression in a murine pregnancy model. Female Balb/C mice ($n=40$) were orally infected with *C. rectus* and/or *P. gingivalis* over a 16-week period and mated once per week. Pregnant mice were sacrificed at embryonic day (E) 16.5 and placentas were collected and analyzed for TLR4 mRNA levels and qualitative protein expression by real time PCR and immunofluorescence. TLR4 mRNA expression was found to be increased in *C. rectus*-infected group (1.98 ± 0.886 fold difference, $P<0.01$, ANOVA) compared to controls. Microscopic analysis of murine placentas showed enhanced immunofluorescence of TLR4 in trophoblasts, mainly in the placental labyrinth layer. Also, combined oral infection with *C. rectus* and *P. gingivalis* significantly reduced the overall fecundity compared to controls (16.7% vs. 75%, infected vs. non-infected mice respectively, $P=0.03$, *Kaplan-Meier*). The results supported an enhanced placental TLR4 expression after oral infection with periodontal pathogens. The TLR4 pathway has been implicated in the pathogenesis of preterm births; therefore the abnormal regulation of placental TLR4 may give new insights into how maternal periodontitis and periodontal pathogens might be linked to placental inflammation and preterm birth pathogenesis.

Keywords

Mice; Periodontitis; Preterm Birth; *Campylobacter rectus*; *Porphyromonas gingivalis*; Toll-like receptors; Placenta

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INTRODUCTION

Preterm birth (birth at ≤ 37 completed gestational weeks) is the major cause of neonatal mortality/morbidity in the world, accounting for up to 75–85% of the early neonatal deaths as well as to high rates of short-term (low birth weight) and long-term adverse sequelae (hearing/learning problems and cerebral palsy) [1;2]. The pathogenesis of preterm birth is thought to be multi-factorial, possibly initiated by multiple mechanisms including infection, uteroplacental ischemia, hemorrhage, stress and other immunologically mediated processes [3]; however, the development of a pro-inflammatory condition is a common effector pathway that centralizes all multiple risk factors [4]. In particular, uterine infections may account for 25–40% of preterm births and they are strongly linked with a pro-inflammatory systemic state. For example, uterine infections are known to upregulate the production of local pro-inflammatory cytokines, metalloproteinases and prostaglandins that lead to membrane weakening, early membrane rupture and uterine contraction initiation [5]. Uterine infections usually take advantage of ascending mechanisms, which are originated from vaginal infections (i.e. *Neisseria gonorrhoeae* or *Ureaplasma urealyticum*) that lead to intrauterine cavity access, decidua colonization, localized inflammation onset (or chorioamnionitis), intraamniotic infection and ultimately fetal infection [6].

Nonetheless, other sources of infection including the oral cavity have been proposed to facilitate the hematogenous transmission of pathogens that affect normal pregnancy development [7]. In particular, periodontal diseases (gingivitis and periodontitis) are part of the most common chronic infections affecting up to 50% of humans [8] and has been found to be an independent putative risk factor for pregnancy-related complications such as preterm births, low birth weight and preeclampsia, after adjusting for other known obstetric risk factors [9;10]. Periodontitis is initiated when specific microorganisms accumulate between the teeth and gums, forming bacterial biofilms commonly known as dental plaque. The body reacts to dental biofilms by activating the oral mucosal inflammatory response that –in some susceptible patients- is unsuccessful in controlling the infection. With time, the inflammatory response remains chronic and ultimately leads to periodontal connective tissue resorption (alveolar bone loss) and tooth loss [11]. During prolonged periodontal inflammation, periodontal pathogens and related virulence factors invade periodontal tissues, enter the blood stream by means of transient bacteremias [12] and disseminate throughout different systemic organs. In fact, important periodontal pathogens have been detected in human placentas of women with preeclampsia [13] and in the amniotic fluid of pregnant women with a diagnosis of premature labor [14] or premature labor with intact membranes [15;16].

Fetal exposure to periodontal pathogens from maternal oral biofilms has also been demonstrated in umbilical cord blood samples from preterm births by detecting maternal immunoglobulin G (IgG) as well as fetal immunoglobulin M (IgM) to one or more specific oral pathogens. In particular, mothers with a low IgG response to *P. gingivalis* combined with a high fetal IgM response to *C. rectus* showed the highest rate of preterm deliveries (66.7%) among 812 deliveries from a cohort study of pregnant mothers (adjusted OR 10.3; $P < 0.0001$) [17], suggesting that *P. gingivalis* and *C. rectus* could act as fetal infectious agents eliciting complications during pregnancy. *C. rectus* is a Gram negative anaerobe and motile bacterium unique to the oral cavity that is phylogenetically related to *H. pylori* and is associated with ulceration of the periodontal attachment apparatus [18]. Interestingly, other *Campylobacter* species are known to be a significant causal agent of sheep and cattle abortion due to a marked tropism for placental tissues [19]. In animal models, *C. jejuni* and *C. fetus* infections result in impaired development and fetal growth restriction (FGR) [20]. We have previously reported that a subcutaneous infection with *C. rectus* in pregnant mice disseminates to placental tissues and induces FGR [21;22], placental inflammation and structural alterations. [23]. Likewise, animal experiments using *Porphyromonas gingivalis* in a subcutaneous infection model have

shown increased maternal inflammatory serum markers (Interleukin-6 and tumor necrosis factor alpha TNF- α), and increased fetal biochemical markers of placental inflammation (prostaglandin E₂) in murine amniotic fluid [24;25].

Placental infection and subsequent inflammation have been associated with preterm labor, so the biological pathways related to early inflammatory responses are likely to mediate pathogenesis. *Toll-like* receptors (TLRs) are pattern recognition receptors that play a key role in the innate inflammatory response [26] and have been proposed to play important roles in pregnancy maintenance, placental immune protection and delivery initiation [27]. To date, a total of 10 human and 12 murine TLRs have been described. In general, TLRs can be categorized into two main groups based on their ligands: the first group consists of TLR1, 2, 4, and 6 which recognize bacterial molecules such as lipopolysaccharide (LPS), lipoteichoic acid and peptidoglycan. The second group consists of TLR3, 7, 8, and 9 that recognize pathogen-associated nucleic acids patterns [28]. Here in we focused on TLR4 which is selectively activated by Gram negative LPS, in conjunction with CD14 [29]. Since *P. gingivalis* and *C. rectus* are Gram negative periodontal pathogens, the main objective of this study was to determine whether an oral infection with *C. rectus* and *C. rectus/P. gingivalis* combined infection could affect fetal growth, fecundity and induce placental inflammation along with enhanced expression of TLR4 in a timed-pregnancy murine model.

METHODS

Timed-pregnancy murine model

Balb/C mice were obtained at 6–8 weeks of age and maintained on a 12-h light/dark cycle (0700 to 1900 light) and a constant temperature of 25°C, receiving distilled water and food *ad libitum*. To facilitate bacterial colonization, all female groups were changed to a soft chow enriched with a dextrose solution (30%) as plaque-promoting diet during infection period. For mating purposes, females were age-matched when 20 weeks old, and males were randomly assigned to experimental groups and remained the same until the end. Female pregnancies were confirmed by the presence of a vaginal plug plus significant weight changes (>1.5 grams gain in a week). Mice were infected daily over a 16-week period and mated once/week. When pregnant, female mice were sacrificed at embryonic day (E) 16.5 and placental tissues were collected and analyzed. All procedures were in accordance with animal guidelines and were approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

Bacterial strains and inoculum preparation

C. rectus 314 and *P. gingivalis* A7436 aliquots were maintained in Wilkins Chalgren anaerobic broth medium (WC broth; DSMZ, Braunschweig, Germany) containing 10% skim milk at –80°C. *C. rectus* aliquots were reconstituted on PRAS ETSA plates (Enriched Tryptic Soy Agar from Anaerobe Systems, Morgan Hill CA) and *P. gingivalis* aliquots on Anaerobic Reducible Blood Agar (from Remel, Lenexa KA). For experiments, bacteria were anaerobically grown under 5% CO₂, 10% H₂–85% N₂ atmosphere at 37°C for 4–6 days. Bacterial suspensions were prepared from primary cultures at their log phase of growth, and concentrations were determined by spectrophotometry (Cecil Instruments, Cambridge, UK) with a measured optical density at 600 nm (*C. rectus*) and 660 nm (*P. gingivalis*) corresponding to 1×10⁹ bacteria/ml respectively. Finally, all oral preparations were adjusted accordingly to keep the same concentration during oral infection experiments.

Oral infection

The oral infection model of experimental periodontitis has been described elsewhere [30]. This model involves a pretreatment phase with antibiotics to suppress the oral flora to permit the

colonization of exogenously applied human oral bacterial strains and the use of carboxymethylcellulose (CMC) as a carrier to facilitate bacterial colonization. Briefly, before experiment baseline 40 female Balb/C mice were pretreated for 4 days with Kanamycin/Ampicillin (50mg/kg-25mg/kg) followed by a 3-day antibiotic wash out period, and then randomly assigned to experimental groups (Table 1 and Figure 1). Mice were topically infected in the oral cavity with 1×10^{10} live bacterial units/100uL in a phosphate-buffered saline (PBS) and 2% CMC preparation on a daily basis. Controls included a blank group (same diet without oral infection) and a negative control group (CMC application without bacteria).

Placental histology and Immunofluorescence

Placentas were fixed in 4% paraformaldehyde, bisected sagittally, processed and embedded in paraffin. Sections (~5 μ m) were stained using hematoxylin/eosin (H&E) for structural analysis; other sections were processed for immunofluorescence. Briefly, tissue antigens were rescued with Safeclear[®] (Fisher Protocol, Fair Lawn NJ) for 20 minutes and washed under serial ethanol concentrations. After washing in 0.2% Triton/PBS, sections were incubated in 10% non-immune goat serum and bovine serum albumin in PBS for 1 hour. Rabbit anti-TLR4 monoclonal antibody (Zymed, Invitrogen, Carlsbad CA) and mouse anti-mouse Cytokeratin 7 monoclonal antibody (RCK105 from Abcam, Cambridge, UK) were incubated overnight on a 1:50 concentration. Cytokeratin 7 was chosen as a trophoblast marker following the recommendations of the workshop report on cell culture models of trophoblasts [31]. After vigorous washing, secondary biotinylated antibodies were applied for 1 hour (Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG from Molecular Probes, Invitrogen, Carlsbad CA) for 1h. Sections were washed in 0.2% Triton/PBS, mounted and coverslipped with Vectashield (Vector labs, Burlingame CA). All stained sections were analyzed and photographed under confocal microscopy (LSM5, Carl Zeiss, Thornwood NY).

Quantitative RT-PCR for TLR4

Total RNA was isolated from all placental tissues (n=135) with the use of the RNeasy Mini Kit (Qiagen). cDNA from 2 μ g of total RNA was synthesized using the Omniscript Kit (Qiagen) and random decamer primers. Real-time PCR was performed with 1 μ L cDNA, TaqMan Universal PCR mix, and 20X primer (Mm00445273_m1 from Applied Biosystems, Foster City, CA), in a 7000 Sequence Detection System (ABI Prism, Applied Biosystems). Reactions were performed in duplicates and in two independent times. The Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) gene was used as an endogenous control (housekeeping gene). Results were evaluated using the delta-delta Ct method, where delta Ct was calculated as (TLR4 Ct) (GAPDH Ct), and the relative quantity of TLR4 mRNA expression was calculated by the delta-delta Ct as $2^{-[(\text{infected sample delta Ct}) - (\text{control sample delta Ct})]}$.

Statistical analysis

A sample size of 8 mice per group was calculated [power (1- β) of >0.90% with alpha-error threshold of (α)=0.05] based on our previous results on fetal growth restriction after *C. rectus* systemic infection [22]. Categorical variables (number of pregnancies and number of fetuses/resorptions per group) were summarized using frequencies and percentages, and continuous variables (fetal weight/length and mRNA levels) were described using means and standard errors. Distributions of fetuses/resorptions were compared using the Chi-square test. Mean fetal weight and length values as well as mRNA levels were compared using Analysis of Variance (ANOVA). To determine whether oral infection correlated with FGR and resorption induction, a general linear regression model (GLM procedure) was used to control for clustering within litters per group. Also, resorptions were considered as zero (value=0) to account for their impact on the average litter weight and length. Kaplan-Meier estimation analysis was used to evaluate cumulative pregnancy events at 16 weeks. Cox proportional

hazards ratios were used to determine the risk of no pregnancies over time after infection. If no significant differences were observed between the control groups (blank and negative), then controls were regrouped as non-infected group for analysis. The threshold for statistical significance was set at a P-value less than 0.05. All analyses were performed using SAS v.9.2. (SAS Institute, Cary, NC).

RESULTS

Oral infection with *C. rectus* and *P. gingivalis* induced growth restricted fetuses and more fetal resorptions

The murine pregnancy events and fetal outcomes after oral infection with periodontal pathogens are depicted in Table 2. The number of mice which became pregnant during the 16 week infection/mating protocol were significantly different among all groups ($P < 0.05$, Chi-square test) as well as in the distribution of fetuses and resorptions ($P < 0.01$). On average, fetuses from infected mice were smaller and lighter, although fetal lengths were not statistically different among all groups ($P = 0.08$, ANOVA). When control groups were compared, no significant differences were observed for number of fetuses/resorptions [27(96.4%) vs. 41 (80.4%), $P = 0.27$, Chi-square], and for fetal weight values [0.47 ± 0.012 vs. 0.39 ± 0.035 , $P = 0.87$, ANOVA, blank and negative groups respectively]. However, when fetal weight values from infected mice were compared to those from non-infected mice (regrouped controls), a statistically significant decrease was observed for the *C. rectus* and *P. gingivalis* infected group ($P < 0.05$, ANOVA, Figure 2). Overall, the general linear regression model which adjusted for clustering within litters was non-significantly associated with FGR induction ($P = 0.28$, GLM procedure).

Murine fecundity was affected after oral infection

Murine fecundity was significantly different among experimental groups (Table 2 and Figure 3). 68.8% of non-infected mice got pregnant; however fecundity decreased to 58.3% in the *C. rectus* infected group and only 16.7% of mice receiving *C. rectus* and *P. gingivalis* combined infection got pregnant. When cumulative pregnancies events were estimated under Kaplan Meier analysis, it was found that the *C. rectus* and *P. gingivalis* group had the lowest fecundity rate (Hazard Ratio 0.19[0.041–0.856], $P = 0.03$) compared to *C. rectus* group (HR 0.87[0.310–2.154], $P = 0.68$) and to non-infected mice.

Oral infection induced placental inflammation and TLR4 expression

Figure 4 presents representative histological findings on murine placentas. In the control placental tissues, there were scattered inflammatory cells present in the maternal decidua of some samples (not shown). In contrast, areas of focal necrosis and increased inflammatory cell infiltrate were apparent in placentas from infected mice (Figure 4A). Under confocal microscopy, the qualitative analysis of murine placentas from infected mice showed enhanced TLR4 immunofluorescence, particularly more evident in labyrinth trophoblasts (Figures 4B–F).

Oral infection increased placental TLR4 mRNA expression

In agreement with the histological observations, mRNA expression of TLR4 receptors was found to be significantly increased in the *C. rectus* group (1.98 ± 0.886 fold difference, $P < 0.01$ ANOVA) as shown in Figure 5. Even though the TLR4 expression was also higher in the *C. rectus* and *P. gingivalis* group, no significant differences were observed when compared to non-infected mice (1.29 ± 0.871 , $P = 0.06$).

DISCUSSION

This study sought to determine the effect of *C. rectus* and *C. rectus/P. gingivalis* oral infection on fetal growth restriction, fecundity and the placental TLR4 expression in a murine model of pregnancy. Previous experimental murine models of infection have examined the role of bacteria and/or isolated virulence factors (i.e. LPS) on preterm birth pathogenesis, reporting that C3HeB/FeJ mice develop up to 71% preterm births after heat-killed *E. coli* intrauterine injection [32]. However not many animal models of infection have addressed the pathogenic role of live oral bacterial infection on preterm births. Experiments with live periodontal bacteria have used subcutaneously-implanted stainless steel coiled chambers as an infection model to study a chronic distant infection in mice and rabbits, as it induces placental inflammation and growth restriction phenotypes (*C. rectus* and *P. gingivalis*) [33]. The periodontal pathogen *Fusobacterium nucleatum* has also been used for intravenous injections in mice, showing subsequent placental inflammation and increased fetal resorptions and stillbirths [34]. Systemic bacterial dissemination of periodontal pathogens has been evidenced in blood, liver, uteri and individual placentas of growth-restricted fetuses [21–25,33]. This report provides evidence that the mixed oral infection model with *C. rectus* and *P. gingivalis* induces FGR and resorptions together with histological evidence of placental inflammation with areas of focal necrosis, enhanced TLR expression and impaired fecundity.

The oral infection model did not significantly correlate with FGR induction, conversely to our previous observations using the chamber model of infection [22]. This finding might be explained in part by the murine immune system ability to fight the oral infection, decreasing bacterial systemic dissemination/exposure protecting the developing fetuses. Particularly, Baker et al. reported that Balb/C mice are able to produce high titers of *P. gingivalis*-specific IgG using the same model of oral infection [35] and we have previously reported that fetal and placental growth may be unaffected in heat killed-*P. gingivalis* pre-immunized rabbits [36]. In addition, the presence of *C. rectus* or *P. gingivalis* DNA was not detected in placentas coming from the oral infection model (data not shown). Nonetheless, overall infected groups had relatively less number of live fetuses, more resorptions and more growth restriction on average (Table 2 and Figure 2). Moreover, fecundity was significantly affected in infected groups (Figure 3). Only 16.7% of mice infected with *C. rectus* and *P. gingivalis* were able to get pregnant and had smaller litter sizes, situation that was not totally unanticipated as our previous observations on *P. gingivalis* infected animals suggested an impairment of fecundity ([37], unpublished observations). Therefore, we hypothesize that the difference in fetal restriction outcomes may be related to the mucosal immune system clearance at the portal of entry and the consequent low bacterial exposure at the placental level.

Even so, we found a placental inflammatory phenotype along with an enhanced TLR4 expression in murine placentas which is suggestive of an active inflammatory response to bacterial exposure. Histologically, placentas from the infected groups showed apparent chronic inflammation (Figure 4A), and the double-staining immunofluorescence analysis showed that TLR4 expression was notably confined to trophoblasts in the labyrinth layer (Figures 4B–F). Furthermore, placental TLR4 mRNA levels showed to be significantly increased almost two-fold in the *C. rectus*-infected group and mildly increased (30%) in the double infection group approaching statistical significance (Figure 5, $P=0.06$). This finding might be explained by the limited number of placentas ($n=12$) available for analysis since only 17% (2 out of 12) mice got pregnant in the combined infection group.

TLRs are highly involved in responding to inflammatory processes in the presence or absence of infection in several tissues. In particular, TLRs are thought to be critical players of the innate immune response during pregnancy, which have significant implications for the success or failure in both early and late gestation [38]. Toll-like receptors expression has been described

in the human placenta, mostly at the dominant cell type: the trophoblast [39–41]. Trophoblasts are the first cells to differentiate from the outer layer of the blastocyst and are believed to participate during endometrial implantation and placental development [42]. Trophoblasts have also been proposed to coordinate the immune response during both processes by regulating immune cells migration (macrophages and NK cells) to the endometrial implantation site through TLRs activation and chemokines production [43–45]. Furthermore, abnormalities in decidual TLRs expression or function have been linked to abnormal placentation, inflammation, and adverse pregnancy outcomes [46]. Interestingly, our data suggest that murine placentas develop pro-inflammatory features after oral infection with periodontal pathogens, where placental trophoblasts notably express more TLR4 as compared to placentas from non-infected mice (Figures 4C–H), and these observations were also consistent with the placental mRNA levels (Figure 5). In fact, TLR4 receptors have been shown to mediate the murine placental inflammatory response and fetal death to *F. nucleatum*, and mice deficient for TLR-4 show protection against bacterial and LPS-induced preterm birth [47]. Moreover, the selective antagonism of TLR-4 inhibits inflammation and preterm uterine contractility in a nonhuman LPS infection model in Rhesus monkeys [48].

The scope of this report was limited to determine TLR4 expression in response to oral Gram negative bacteria as proof of principle; however it is possible that other type of TLRs participate during the placental immune responses. For example, *C. rectus* offers a wide array of virulence factors including surface layer (S-layer) proteins [49], cytolethal distending toxin (CDT) [50], GroEL-like proteins [51] and lipopolysaccharide (LPS)[52]. In addition, *P. gingivalis* also possess a plethora of virulence factors including capsule, fimbriae, proteases (Gingipains) and LPS [53]. Therefore, either *C. rectus* or *P. gingivalis* might be able to induce placental inflammation via different virulence factors that could potentially be sensed by different TLRs including TLR-2 (peptidoglycans or fimbriae), TLR4 (LPS) or TLR-5 (flagellin).

In conclusion, previous models used to mimic chronic infections in mice result in dissemination of live oral bacteria to the placental tissues and impair fetal growth. However, this mixed oral infection model combines two commensal and critical pathogens that in combination are strongly associated with periodontal disease in humans [54]. Therefore, it is plausible to speculate that chronic systemic exposure to oral bacteria and related virulence factors may affect early pregnancy via pro-inflammatory mechanisms and such hypotheses warrant further investigations [55]. Further research is also needed to characterize the placental TLR response to oral pathogens in humans to elucidate and validate pathogenic mechanisms.

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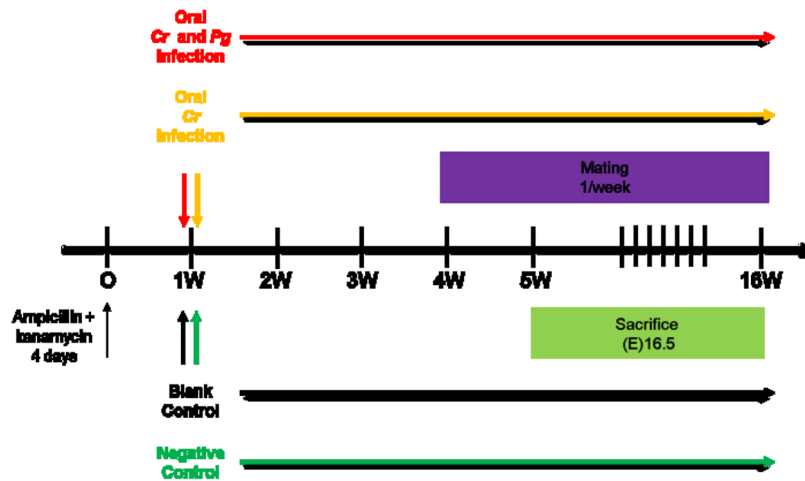


Figure 1. Experiment timeline

Female (3-month old) Balb/C mice were pretreated for 4 days with Kanamycin/Ampicillin (50mg/kg-25mg/kg) and then randomly assigned to experimental groups. Mice were infected daily over a 16-week period and mated once/week. When pregnant, mice were sacrificed at embryonic day (E)16.5 and placental tissues were collected and analyzed for TLR4 expression by immunofluorescence (confocal microscopy) followed by RT-PCR. Cr=*C. rectus*; Pg=*P. gingivalis*; CMC=carboxymethylcellulose.

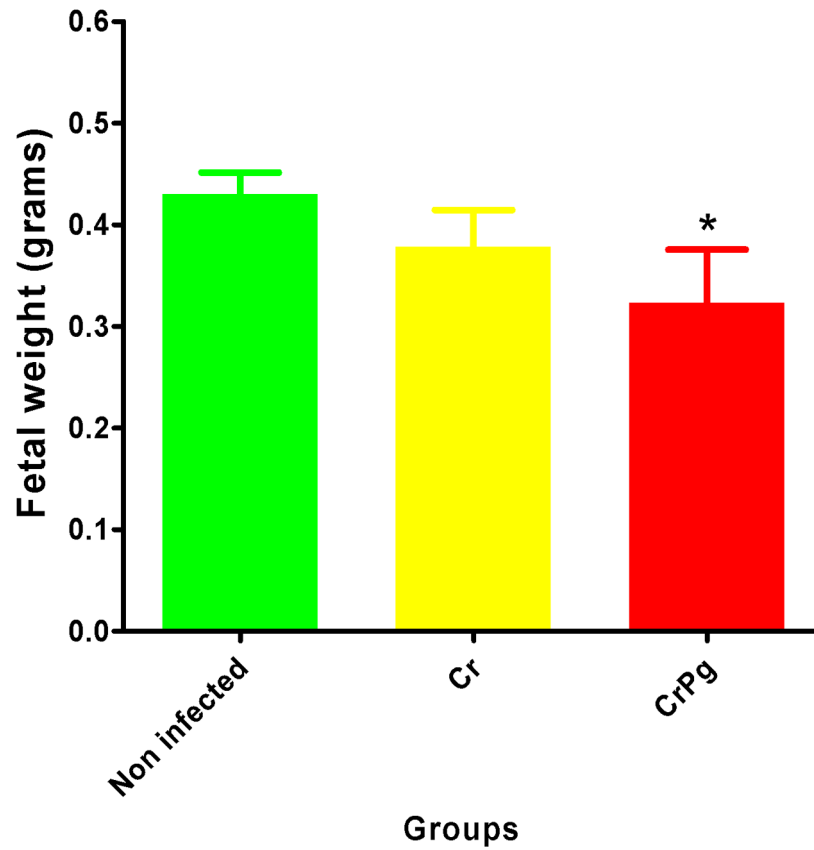


Figure 2. Oral infection induces low weight fetuses

Averaged fetal weight values from infected mice were smaller when compared to those from non-infected mice. Columns and bars represent means and standard errors. Cr=*C. rectus*; Pg=*P. gingivalis*; * $P < 0.05$, ANOVA.

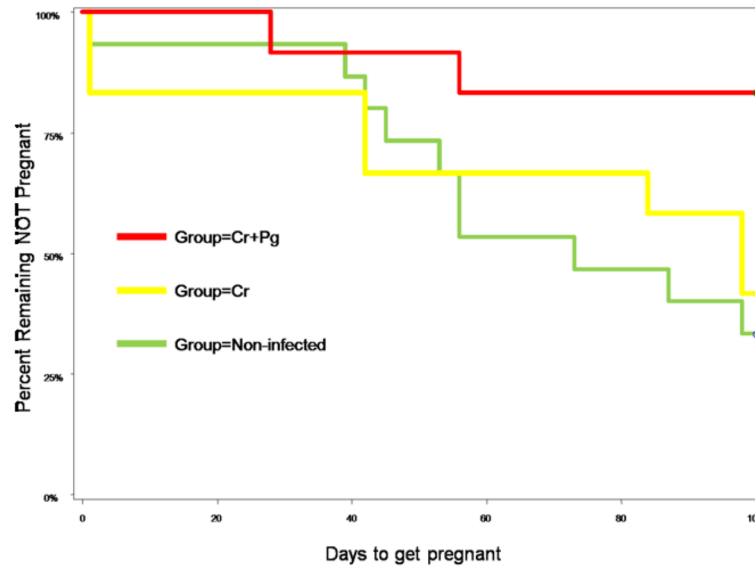


Figure 3. Oral infection affects murine fecundity

Kaplan Meier Analysis - *C. rectus* and *P. gingivalis* infected mice had the lowest fecundity rate (16.7%, HR 0.19[0.041–0.856], P=0.03, Kaplan-Meier) compared to *C. rectus* (58.3%, Hazard Ratio 0.87[0.310–2.154], P=0.68) and to non-infected mice.

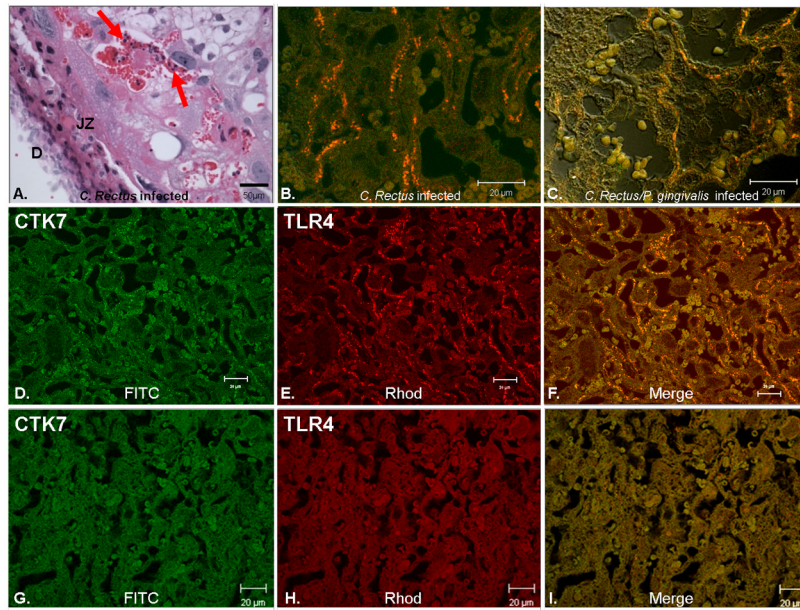


Figure 4. Oral infection induced inflammation and TLR4 expression in murine placentas
 Histological and immunofluorescence analysis of murine placentas. 4A: Representative image depicting extensive junctional zone (JZ) along with an increased inflammatory cell infiltrate (arrowheads) in placentas from infected mice. Decidua (D); Bar=50 μ m. 4B–4C: immunofluorescence of a placenta from the *C. rectus* and *C. rectus/P. gingivalis* infected groups respectively. Images represent 3 merged channels (Rhodamine, FITC and DIC) depicting trophoblasts expressing TLR4 from both groups. 4D–4E: images depict the expression of Cytokeratin 7 (FITC-green) and TLR4 (Rhodamine-red) in labyrinth trophoblasts from a different *C. rectus*-infected section; 4F: image illustrates co-localization of Cytokeratin 7 and TLR4 suggesting enhanced expression of TLR4 by labyrinth trophoblasts. 4G–4I: images correspond to fluorescence negative controls (no primary antibodies used) for Cytokeratin 7 and TLR4 respectively. Rhod=Rhodamine (red); FITC= Fluorescein isothiocyanat (green); White bars=20 μ m.

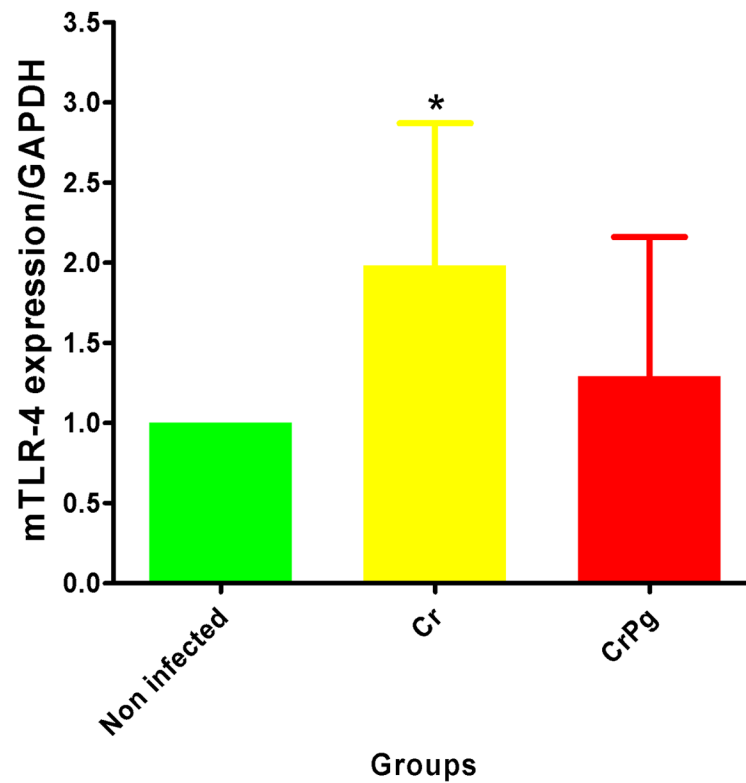


Figure 5. Oral infection with periodontal pathogens increased TLR4 mRNA expression in murine placentas

After normalization, TLR4 mRNA levels were significantly increased in *C. rectus* group placentas (1.98 ± 0.886 fold difference, $P < 0.01$). Although TLR4 mRNA levels were also higher in the combined infection group, no significant differences were observed when compared to non-infected mice (1.29 ± 0.871 , $P = 0.06$). Cr=*C. rectus*; Pg=*P. gingivalis*; * $P < 0.01$, ANOVA).

Experimental groups

All groups were given *ad libitum* soft chow enriched with a dextrose solution (30%) as plaque-promoting diet during 16 weeks of experimentation. CMC=carboxymethylcellulose; CFU=colony forming units.

Table 1

Experimental Group	N	Description
Blank control	8	High-glucose soft chow
Negative control	8	High-glucose soft chow CMC topical application
<i>Campylobacter rectus</i> infection	12	High-glucose soft chow CMC + <i>C. rectus</i> (1×10^{10} CFU)
<i>Campylobacter rectus</i> and <i>porphyromonas gingivalis</i> infection	12	High-glucose soft chow CMC + <i>C. rectus</i> (1×10^{10} CFU) CMC + <i>P. gingivalis</i> (1×10^{10} CFU)

Table 2**Pregnancy events and fetal outcomes after oral infection**

Distribution of pregnancies, number of fetuses and resorptions were significantly different among groups ($P < 0.01$, *Chi square*). Fetuses from infected mice were smaller and lighter on average, but fetal lengths were not different among all groups ($P = 0.08$, ANOVA). CMC=carboxymethylcellulose; CFU=colony forming units.

Experimental Group	N	Pregnancy events [n (%)]	Fetuses [n (%)]	Resorptions [n (%)]	Fetal length (mm) (mean±SE)	Fetal weight (grams) (mean±SE)
Non-infected	16	11(68.8)	68(86.1)	11(13.9)	1.28±0.060	0.43±0.021
<i>C. rectus</i>	12	7(58.3)	55(73.3)	20(26.7)	1.09±0.104	0.37±0.036
<i>C. rectus</i> and <i>P. gingivalis</i>	12	2(16.7)	12(70.6)	5(29.4)	1.02±0.265	0.32±0.052*