

Porphyromonas gingivalis Infection in Pregnant Mice Is Associated with Placental Dissemination, an Increase in the Placental Th1/Th2 Cytokine Ratio, and Fetal Growth Restriction

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Our previous animal studies showed that maternal *Porphyromonas gingivalis* infection in a subcutaneous chamber is associated with hepatic and uterine translocation, as well as systemic induction of maternal inflammatory responses, both of which were associated with fetal growth restriction (FGR). However, *P. gingivalis*-challenged dams had fetuses with either FGR (2 standard deviations below mean weight of nonchallenged dams) or normal weight. Therefore, the objective of this study was to determine whether maternal infection with *P. gingivalis* compromises normal fetal development via direct placental invasion and induction of fetus-specific placental immune responses characterized by a proinflammatory Th1-type cytokine profile. *P. gingivalis*-specific DNA was detected in placentas and fetuses of FGR and normal littermates from *P. gingivalis*-infected dams. Th1- and Th2-type cytokine mRNA as well as tumor necrosis factor alpha and transforming growth factor β 2 mRNA were examined in placental tissue by using reverse transcription-PCR to determine Th1/Th2 ratios. For eight litters containing both normal-weight and FGR fetuses, *P. gingivalis* DNA was detected only in the placentas of FGR fetuses. All fetuses and all amniotic fluid samples from infected and control dams were negative for *P. gingivalis* DNA. mRNA levels of gamma interferon and interleukin-2 (IL-2) were significantly increased in placentas of FGR fetuses, while expression of IL-10 was significantly decreased in the same group. These data indicate that, in *P. gingivalis*-challenged dams, within each litter there is placenta-specific translocation of *P. gingivalis* that results in growth restriction of the targeted fetus, which is associated with a shift in the placental Th1/Th2 cytokine balance.

Periodontitis is a chronic infectious disease, and the primary etiological agents are gram-negative, anaerobic bacteria that occupy the tooth-associated biofilm in the subgingival plaque. Among them, *Porphyromonas gingivalis* is thought to be a major periodontal pathogen (1, 2). Human studies in patients with periodontitis and animal studies have suggested *P. gingivalis* may be an important component in the underlying association in linking periodontitis to preterm birth (6). Patients with periodontal diseases show that *P. gingivalis*-specific sensitized T cells are present in the peripheral blood, indicating systemic exposure to *P. gingivalis* (7, 22). Our previous animal studies showed that maternal *P. gingivalis* infection in a subcutaneous chamber is associated with hepatic and uterine translocation, and this dissemination is correlated with fetuses that exhibit fetal growth restriction (FGR) (15).

A successful pregnancy is thought to be characterized by a dominant Th2-type immunity with predominance of interleukin-4 (IL-4) and IL-10 expression over tumor necrosis factor alpha (TNF- α), gamma interferon (IFN- γ), and IL-2 in the placenta (28). Th2-type cytokines are associated with humoral immunity, which is known to favor implantation, induce and

maintain allograft tolerance, and control harmful maternal responses (13). Th1-type cytokines induce several cytotoxic and inflammatory reactions which are known to be potentially deleterious for the conceptus by modulating the integrity of the placental membrane and uterine tone (20, 21, 29, 30, 32). For example, TNF- α is believed to induce macrophage matrix metalloproteinases to weaken the placental membrane and release prostaglandin E2, which induces uterine constriction (4, 33). The administration of TNF- α and IFN- γ to pregnant mice increases fetal abortion and fetal abnormalities such as reduction of fetal weight and retardation of eye development (34). Thus, infectious challenges that trigger abnormal pregnancies may be mediated by changes in the placental Th1/Th2 balance. In addition, transforming growth factor β 2 (TGF- β 2) is a potent immunosuppressive factor and can help to reduce activation of NK cells by potentially abortive events, such as intrauterine exposure to lipopolysaccharide or to interferons. It has been reported that TGF- β 2 production is defective in women with repeated miscarriages (14).

Our previous studies (17, 24–26) and recent work in a murine model (15) showed that *P. gingivalis* infection can result in hepatic and uterine dissemination, triggering growth restriction of selected fetuses. Since not all fetuses within litters were affected, we hypothesized that translocation of *P. gingivalis* or its components into the placenta may induce local immune responses that impair placental function, thereby mediating FGR. We further hypothesized that *P. gingivalis*-associated

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immune responses would be characterized by a shift of placental antiinflammatory Th2-type immunity to proinflammatory Th1-type immunity and, consequently, induce the occurrence of pregnancy complications, such as FGR and fetal death.

MATERIALS AND METHODS

Animal experiments. A mouse subcutaneous chamber model was used to establish a localized infection with *P. gingivalis* strain A7436. Animal husbandry and bacterial preparation for inoculation are described in detail in Lin et al. (15). Briefly, mice were implanted with stainless steel chambers and immunized with 10^9 CFU of heat-killed *P. gingivalis*. Female mice were bred with the same-strain male mice and challenged with 10^7 CFU of live *P. gingivalis* on gestation day (GD) 7.5, while the control group received broth. On GD 16.5, animals were sacrificed and pregnancy outcomes were assessed. FGR was defined as fetuses with weights 2 standard deviations (SD) smaller (i.e., less than 0.46 g) than normal fetal weight (NFW; 0.56 ± 0.05 g). Amniotic fluid was collected from each fetal-placental unit and pooled per pregnant animal. Placentas and fetuses were collected, washed with sterile saline solution, and stored at -80°C for future use.

mRNA expression of cytokines in placenta. mRNA expression of cytokines was analyzed from all placentas from FGR fetuses and their littermates and from selected placentas (numbers 1, 3, 5, 7, etc.) from the control group and the *P. gingivalis*-challenged group with all normal-weight fetuses. Total RNA from each placenta was extracted using Tri reagent (Sigma, St. Louis, Mo.) according to the manufacturer's instructions. Briefly, placental samples were cut into half, and one half was homogenized and digested in 500 μl of Tri reagent for 5 min at room temperature to ensure complete dissociation of nucleoprotein complexes. After adding 100 μl of chloroform with vigorous shaking, samples were centrifuged at $12,000 \times g$ for 15 min at 4°C , which separated the mixture into three phases. The aqueous phase containing RNA was collected and mixed with 250 μl of isopropanol. After centrifugation, the supernatant was removed and the RNA pellet was washed with 75% ethanol. After drying the pellet for 10 min with air, 10 μl of DNase-RNase-free water was added to dissolve the RNA pellet. The concentration of each RNA sample was adjusted to 1 $\mu\text{g}/\mu\text{l}$. cDNA was synthesized using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech). Briefly, 1 μg of total RNA was reverse transcribed in a total volume of 15 μl using cloned FPL CpureMurine reverse transcriptase, and first-strand cDNA was synthesized using *NotI*-d(T)₁₈ bifunctional primer according to standard procedures. Specific primers were used for PCR to obtain double-stranded DNA (amplicon) of IFN- γ (8), IL-2 (11), IL-12 (12), IL-4 (23), IL-10 (19), TNF- α (10), TGF- β 2 (31), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, a housekeeping gene) for each sample.

Following PCR amplification, amplicons (10 μl) were separated by 2% Trisacetate ($1 \times \text{TAE}$) agarose gel electrophoresis and stained with 0.5 μg of SYBR Gold (Molecular Probes, Eugene, Oreg./ml). We used LumiAnalyst combined with Image Analysis software version 3.1 (Boehringer Mannheim GmbH, Mannheim, Germany) to measure the relative intensities of bands corresponding to specific amplicons. Relative levels of mRNA were expressed by calculating the ratio of each band's intensity (i.e., specific DNA of each sample) normalized to the intensity of the GAPDH signal.

***P. gingivalis* detection.** All placentas from FGR fetuses and their littermates were analyzed for *P. gingivalis* DNA. Two placentas selected from control dams and selected placentas (numbers 1, 3, 5, 7, etc.) from *P. gingivalis*-challenged dams with all normal-weight fetuses were analyzed. DNA from placental tissue of NFW and FGR fetuses or whole fetuses was extracted for the detection of *P. gingivalis* as described elsewhere (15). Each placenta or fetus was scored as positive or negative for the presence of *P. gingivalis* DNA detected by PCR. Positive controls included *P. gingivalis* alone or tissues spiked with exogenous *P. gingivalis*. The detection limit for *P. gingivalis* alone was 10 CFU/PCR, and the detection limit for the *P. gingivalis*-spiked tissues was 100 CFU/PCR (data not shown). Six representative amniotic fluid samples from dams with FGR were cultured (as described previously in reference 15) to determine whether live bacteria were present.

Statistical analysis. To determine whether fetal exposure to *P. gingivalis*, as determined by *P. gingivalis* DNA in placental samples, was correlated with FGR, exact logistic regression models were used to control for clustering of fetuses within dams. Similarly, the effects of *P. gingivalis* on mean fetal weight were adjusted for litter-dam clustering using mixed models with the dam as a random stratification factor, with a *P* value of less than 0.05 as statistically significant. In addition, statistically significant differences in placental cytokine mRNA expression were determined by conditional logistic regression with exact inference for

small sample size to control for clustering of mouse pups within dams by treating the dam as a factor in a stratified model (16, 27). Relative cytokine expression for individual cytokines and for Th1 and Th2 groups of cytokines was compared separately for placentas of fetuses in challenged versus control dams and for growth-restricted fetuses versus normal-weight fetuses among challenged dams using mixed models with the dam as a random stratification factor. Results are presented as mean values \pm the standard error of the mean for the different groups of dams. Data were analyzed using Statistical Analysis Software (SAS Institute, Cary, N.C.).

A Z score transformation was completed to normalize Th1 or Th2 profiles and to determine relative shifts in the overall Th1/Th2 ratio. Individual cytokine values for each placenta within each dam were transformed into Z scores (equation 1). Individual Z scores for each individual cytokine were averaged for the placentas to arrive at a cytokine-specific Z score for each dam (equation 2). Z scores for Th1- and Th2-type cytokines were computed for dams by averaging the Z scores of Th1 (IFN- γ and IL-2 plus IL-12) (equation 3) and the Z scores of Th2 (IL-4 plus IL-10) (equation 4).

$$Z_{\text{cytokine specific for each placenta}} = \frac{X_i - X_{\text{mean}}}{\text{SD}} \quad (1)$$

where X_i is the relative intensity of a specific cytokine mRNA over GAPDH intensity in an individual placenta, X_{mean} is the mean relative intensity of a specific cytokine mRNA in all placental samples, and SD is the standard deviation of the mean of relative intensity of a specific cytokine mRNA in all placental samples.

$$Z_{\text{cytokine specific for each dam}} = \text{Average of } Z_{\text{cytokine specific for each placenta}} \quad (2)$$

$$Z_{\text{Th1}} = \text{Average of } (Z_{\text{IFN-}\gamma} + Z_{\text{IL-2}} + Z_{\text{IL-12}}) \quad (3)$$

$$Z_{\text{Th2}} = \text{Average of } (Z_{\text{IL-4}} + Z_{\text{IL-10}}) \quad (4)$$

RESULTS

Exposure of mice to *P. gingivalis* during pregnancy resulted in two possible outcomes for the litters. Either all fetuses were normal weight (NFW), or one or more fetuses in the litter were low weight (FGR). Because abortifacient or teratogenic agents seldom have 100% penetrance, a litter with both normal and low-weight fetuses provides an opportunity to examine differences at the placental and fetal levels. These groups help distinguish effects mediated through maternal mechanisms or placental and fetal mechanisms. Consequently, placental results were divided into four groups: group I, placentas from control dams (36 placentas from 8 dams); group II, placentas from *P. gingivalis*-challenged dams with all normal-weight fetuses (32 placentas from 12 dams); group III, placentas of normal-weight fetuses from *P. gingivalis*-challenged dams with both normal- and low-weight fetuses within the litter (18 placentas from 5 dams); and group IV, placentas of low-weight fetuses from *P. gingivalis*-challenged dams with one or more low-weight fetuses within the litter (30 placentas from 8 dams).

Detection of placental *P. gingivalis* by PCR. In the broth-challenged control group (group I), all placental samples examined (two per mouse) were negative for *P. gingivalis* DNA (data not shown). In the 20 *P. gingivalis*-challenged animals, eight dams had one or more FGR fetuses. Overall, there was a total of 107 fetuses scored as *P. gingivalis* positive or negative and as FGR present or absent (Table 1). For the 31 placentas from FGR fetuses, 39% had positive PCR signals for *P. gingivalis* and 61% were *P. gingivalis* negative (Table 1). All of the 76 NFW fetuses had placentas that were also *P. gingivalis* negative.

The mean fetal weight of the FGR *P. gingivalis*-positive group was significantly lower than that of the FGR *P. gingivalis*-negative and NFW groups (Table 1). In addition, the fetal

TABLE 1. Average fetal weight and PCR detection of *P. gingivalis* DNA in placentas from NFW and FGR fetuses among *P. gingivalis*-challenged dams

Fetal growth	Presence of PCR signal for <i>P. gingivalis</i> DNA	No. of fetuses	Mean fetal wt ± SD (g)
FGR ^a	Yes	12	0.27 ± 0.09 ^c
	No	19	0.39 ± 0.04 ^d
NFW ^b	Yes	0	
	No	76	0.55 ± 0.03

^a FGR, fetal growth restriction.
^b NFW, normal fetal weight.
^c Significantly different from FGR *P. gingivalis* negative and NFW ($P < 0.001$; mixed models with the dam as a random stratification factor).
^d Significantly different from NFW ($P < 0.001$; mixed models with the dam as a random stratification factor).

weight of the FGR *P. gingivalis*-negative group was also significantly less than that of the NFW fetuses (Table 1). As an example, Fig. 1 shows the *P. gingivalis* distribution in placental tissues of a representative *P. gingivalis*-challenged dam. PCR signals from placental tissue taken from littermates (two NFW, two FGR) are shown. Note that no *P. gingivalis* amplicon signal was present for the two NFW fetuses, but signal was present for both FGR fetuses. The association between *P. gingivalis* signal in placenta and FGR was statistically significant at a P level of <0.001 (Table 1). In contrast, there was no detectable *P. gingivalis* PCR signal in FGR or NFW fetuses, nor was there live *P. gingivalis* detected in the anaerobic culture of amniotic fluid from either FGR or NFW fetuses (data not shown).

Placental mRNA expression of Th1- and Th2-type cytokines.

The relative expressions of selected genes (Th1 cytokines IFN- γ , IL-2, and IL-12; Th2 cytokines IL-4 and IL-10; a proinflammatory cytokine, TNF- α ; and an immunosuppressive cytokine, TGF- β 2) in placenta are shown in Table 2. When cytokine mRNA expression in placentas from control dams (group I) was compared with that in placentas from all challenged dams collectively (groups II, III, and IV), IFN- γ was increased from 1.8 to 5.7% ($P = 0.0031$) and IL-2 was increased from 0.2 to 3.5% ($P = 0.0007$). In contrast, mRNA

TABLE 2. Relative intensity of cytokine mRNA expression in placentas of control and *P. gingivalis*-challenged groups^a

Cytokine	Relative mRNA expression in group (no. of placentas analyzed)			
	I (36)	II (32)	III (18)	IV (30)
Th1				
IFN- γ	1.8 ± 1.1	3.7 ± 1.1	3.4 ± 0.9	11.5 ± 1.4 ^{b,c}
IL-2	0.2 ± 0.8	2.3 ± 0.6	2.8 ± 0.7	5.2 ± 1.0 ^{b,c}
IL-12	19.1 ± 4.6	14.5 ± 5.3	13.2 ± 3.9	30.9 ± 5.3 ^c
Th2				
IL-4	17.1 ± 2.6	8.4 ± 3.1	8.9 ± 1.9	4.7 ± 2.7 ^b
IL-10	18.3 ± 5.9	27.7 ± 5.8	20.5 ± 5.6	7.5 ± 7.0 ^{b,c}
TNF- α	13.9 ± 5.1	10.6 ± 6.8	18.9 ± 4.8	16.5 ± 6.3
TGF- β 2	59.8 ± 10.8	24.6 ± 9.1	19.3 ± 8.9	14.9 ± 9.2 ^b

^a Relative intensity was calculated as the specific cytokine/GAPDH ratio (represented by percentage) conditioning on dam. Group I, placentas from 8 control dams; group II, placentas from 12 *P. gingivalis*-challenged dams with all normal-weight fetuses; group III, placentas of normal-weight fetuses from 5 *P. gingivalis*-challenged dams with both normal- and low-weight fetuses in litter; group IV, placentas of low-weight fetuses from 8 *P. gingivalis*-challenged dams with one or more low-weight fetuses.
^b $P < 0.05$ compared with group I (statistical test).
^c $P < 0.05$ compared with group III (statistical test).

expression for IL-4 was decreased from 17.1 to 7.1% ($P = 0.0022$) and TGF- β 2 decreased from 59.8 to 18.5% ($P = 0.0064$). These data are consistent with our hypothesis that live *P. gingivalis* infection is associated with increases in inflammatory Th1-type cytokines (IFN- γ and IL-2) and decreases in antiinflammatory Th2-type (IL-4) and immunosuppressive (TGF- β 2) cytokines. To further examine the relationship between changes in Th1- and Th2-type cytokines after challenge with *P. gingivalis*, the challenged group was subdivided into groups II, III, and IV based on fetal weights (Table 2). When cytokine mRNA expression in placentas of FGR fetuses (group IV) was compared with that in their littermates (group III), all Th1-type cytokines examined (IFN- γ , IL-2, and IL-12) increased significantly ($P < 0.05$), while IL-10 decreased significantly ($P = 0.05$). When cytokine mRNA expression in placentas of FGR fetuses (group IV) was compared with that in the control group (group I), Th1-type cytokines (IFN- γ and IL-2) were significantly increased while Th2-type cytokines (IL-4 and IL-10) were significantly decreased ($P < 0.05$).

Changes in the Th1/Th2 ratio after *P. gingivalis* challenge in placentas of low-weight fetuses. The average Z scores of Th1- or Th2-type cytokines were used to demonstrate the relative balance in the placental Th1 and Th2 immunity after *P. gingivalis* challenge (Fig. 2). In the control group, Th2 was dominant in the placenta (Fig. 2, group I). However, following *P. gingivalis* challenge, Th2-type cytokines were lower while Th1-type cytokines were higher, with a loss in Th2 dominance (Fig. 2, groups II and III). Although normal-weight fetuses in the challenged group maintained a similar placental Th1/Th2 profile as those in the control group, growth-restricted fetuses demonstrated an inversion in the cytokine ratio, with a shift to a predominant Th1 cytokine profile (Fig. 2, group IV).

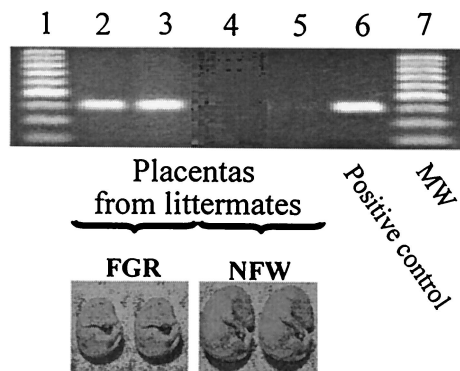


FIG. 1. PCR detection of *P. gingivalis* DNA in placentas from NFW and FGR littermates. *P. gingivalis* DNA PCR signals were detected in placentas from two FGR fetuses, but not in placentas from their two normal-weight littermates (NFW). Representatives of NFW and FGR fetuses are shown below.

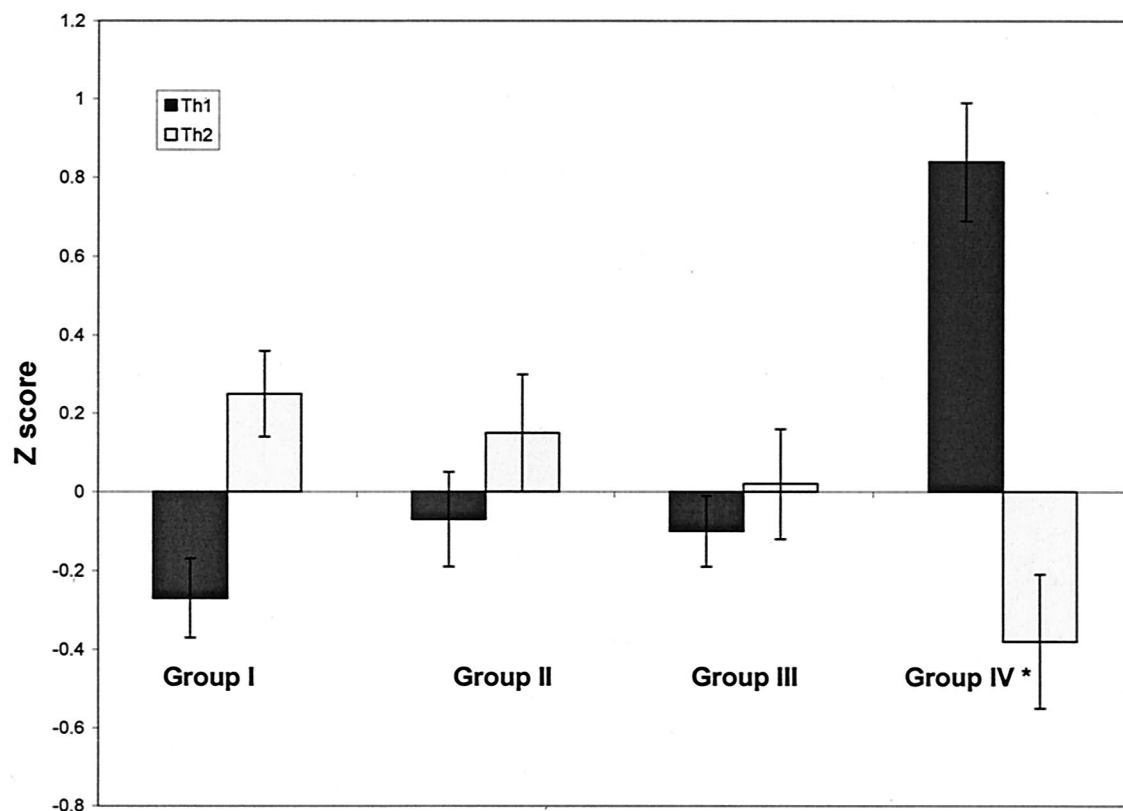


FIG. 2. Average Z scores of mRNA expression of Th1- or Th2-type cytokines in placentas from different treatment and fetal-weight groups. Group I, placentas from fetuses from control dams; group II, placentas from *P. gingivalis*-challenged dams with all normal-weight fetuses; groups III and IV, placentas from NFW fetuses (group III) or FGR fetuses (group IV) from *P. gingivalis*-challenged dams with at least one FGR fetus in their litters.

DISCUSSION

These data show that maternal *P. gingivalis* challenge can result in *P. gingivalis* translocation to placental tissue and induction of FGR, while nonaffected littermates show no evidence of *P. gingivalis* signals in their corresponding placentas. However, *P. gingivalis* signal was not detected in 61% of placentas from FGR fetuses. The absence of a PCR signal may be a false-negative finding due to lack of sensitivity, which may be overcome in future studies using nested PCR. Alternatively, the effect on fetal growth may not require placental translocation, as there are effects on circulatory levels of maternal cytokines following *P. gingivalis* challenge that may impair fetal growth, such as an increase in maternal serum TNF- α levels (15).

The placenta is a rich source of a variety of cytokines of both maternal and fetal origins which have an essential role in maintaining pregnancy and inducing parturition in humans and animals. Studies investigating ascending vaginal-cervical infection and adverse pregnancy outcomes have associated changes in placental cytokine levels with premature rupture of membranes and preterm labor (3). Complicated pregnancy outcomes (e.g., FGR, abortion, and preeclampsia) are associated with increased placental proinflammatory Th1-type cytokines and/or diminished expression of antiinflammatory Th2-type cytokines (20, 21, 30). For example, TNF- α stimulates the

programmed death of human primary villous trophoblast cells, and IFN- γ augments TNF-mediated killing of trophoblasts (35). In addition, the induction of major histocompatibility complex class I and II molecules on the surface of trophoblast cells by IFN- γ presents fetal antigens to maternal T cells and strengthens cell-mediated immune responses, leading to fetal rejection (36). Antiinflammatory cytokines (e.g., IL-4 and IL-10) produced by the placenta have been suggested to play a vital role in the survival to term of the fetal allograft by counteracting deleterious inflammatory cytokines (e.g., TNF- α and IFN- γ), which would ordinarily be expected to protect an individual from tissue with foreign antigens (5, 9, 18, 29).

P. gingivalis infection in this model appears to induce a placental shift in Th1 and Th2 cytokine expression (Table 2). *P. gingivalis* challenge that resulted in FGR was associated with a 6.4-, 26-, and 1.6-fold increase in placental IFN- γ , IL-2, and IL-12, respectively, as well as a 3.6- and 2.5-fold decrease in IL-4 and IL-10, respectively, compared to the control group. These findings parallel the observed shifts in Th1 and Th2 associated with vaginal infection.

Previous work with this model (15) demonstrated that *P. gingivalis* challenge induces maternal immune responses, as shown by an elevation in the serum proinflammatory cytokine TNF- α and a decrease in the antiinflammatory cytokine IL-10. These systemic changes have been associated with FGR. Our

TABLE 3. Summary of major changes occurring in dams, fetuses, and placentas following *P. gingivalis* challenge of pregnant mice

Maternal group ^a	Maternal serum cytokine levels	Maternal serum IgG levels	Fetal weight	Placental changes
Pg- NFW	Normal	Normal	All normal weight (group I)	Th2 dominance
Pg+ NFW	Normal	Normal	All normal weight (group II)	Trend for lower Th2 and higher Th1 responses
Pg+ FGR	Significant TNF- α increase and significant IL-10 decrease	Significant increase	Normal weight (group III)	Trend for lower Th2 and higher Th1 responses
	Significant TNF- α increase and significant IL-10 decrease	Significant increase	Growth restricted (group IV)	Th1 dominance

^a See the text for descriptions of maternal groups. Pg-, *P. gingivalis* negative; Pg+, *P. gingivalis* positive.

findings here, suggesting that the translocation of *P. gingivalis* into the placenta was associated with the incidence of FGR, were also consistent with the changes of pro- and antiinflammatory cytokine mRNA expression in the placentas. These observations indicate that the disseminated organism can induce placental inflammation. However, we found no evidence that *P. gingivalis* was present in either the fetus or the amniotic fluid samples obtained from *P. gingivalis*-challenged dams by PCR or culture methods (data not shown). Furthermore, when amniotic fluid was analyzed using enzyme-linked immunoassays for the cytokines listed in Table 2, amniotic fluid showed no detectable changes in cytokine concentrations (data not shown). Therefore, our data suggest that placental immune and inflammatory responses are correlated with consequent abnormal pregnancy outcomes and may result from placental invasion by *P. gingivalis* and/or its virulence factors, or from the maternal inflammatory responses to *P. gingivalis* challenge.

Our findings are consistent with the concept that *P. gingivalis* infection suppresses expression of protective Th2 cytokines as well as the immunosuppressive cytokine TGF- β 2. When *P. gingivalis* also results in FGR, there is a specific enhancement of Th1 cytokine profiles not seen in the placentas from normal-weight fetuses, even in littermates of FGR fetuses. These data suggest that FGR occurs only when there is a dual effect of Th2 suppression and Th1 enhancement.

In conclusion, in the mouse chamber model, localized *P. gingivalis* infection can disseminate to the placenta and is associated with a shift in placental Th2-type humoral immunity to Th1-type cellular immunity and also FGR or fetal death (15). After *P. gingivalis* challenge, several changes occur in both maternal and fetal tissues that are correlated with FGR fetuses. Table 3 summarizes the changes reported from our previous work (15) as well as the present study. These results support the hypothesis that proinflammatory Th1 cytokine dominance during pregnancy is correlated with adverse pregnancy outcomes. The data suggest that as long as antiinflammatory Th2-type cytokines maintain a balance with proinflammatory Th1-type cytokines in the placenta, the fetus is normal weight even in litters containing FGR littermates.

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