molecular oral microbiology

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Toll-like receptor 4 mediates intrauterine growth restriction after systemic *Campylobacter rectus* infection in mice

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SUMMARY

Campylobacter rectus **is associated with fetal exposure and low birthweight in humans. *C. rectus* also invades placental tissues and induces fetal intrauterine growth restriction (IUGR) in mice, along with overexpression of Toll-like receptors (TLR4), suggesting that TLR4 may mediate placental immunity and IUGR in mice. To test this hypothesis we examined the effect of *in vitro* TLR4 neutralization on trophoblastic proinflammatory activity and studied the IUGR phenotype in a congenic TLR4-mutant mouse strain after *in vivo C. rectus* infection. Human trophoblasts were pretreated with TLR4 neutralizing antibodies and infected with *C. rectus*; proinflammatory cytokine production was assessed by cytokine multiplex assays. Neutralizing TLR4 antibodies significantly impaired the production of proinflammatory cytokines in trophoblastic cells after infection in a dose-dependent manner. We used a subcutaneous chamber model to provide a *C. rectus* challenge in BALB/cAnPt ($TLR4^{Lps\text{-}d}$) and wild-type (WT) females. Females were mated with WT or $TLR4^{Lps\text{-}d}$ males once/week; pregnant mice were infected at (E)7.5 and sacrificed at (E)16.5 to establish IUGR phenotypes. Maternal *C. rectus* infection significantly decreased fetal weight/length in infected WT when compared with sham WT controls ($P < 0.05$, analysis of variance). However, infected $TLR4^{Lps\text{-}d-/-}$ mice did not show statistically significant differences in fetal weight and length when compared with WT controls ($P > 0.05$). Furthermore, heterozygous $TLR4^{Lps\text{-}d+/-}$ fetuses showed IUGR phenotype rescue. We conclude that TLR4 is an important mediator of trophoblastic proinflammatory responses and TLR4-deficient fetuses do not develop IUGR phenotypes after *C. rectus* infection, suggesting that placental cytokine activation is likely to be mediated by TLR4 during low birthweight/preterm birth pathogenesis.**

INTRODUCTION

Periodontal diseases (gingivitis and periodontitis) are among the most common infectious diseases affecting up to 50% of Americans (Albandar, 2002). As a

chronic infection in nature, periodontitis exposes the host to microbial challenge for extended periods of time leading to a persistent oral inflammatory response that ultimately causes alveolar bone resorption and tooth loss. Concomitantly, the susceptible host is exposed to repeated bacteremias and systemic inflammatory mediators that have been shown to contribute to the pathogenesis of some systemic diseases including atherosclerosis and cardiovascular diseases (Beck & Offenbacher, 2005). Periodontitis has also been associated with an increased risk for preterm delivery (PTD) and pre-eclampsia in different human populations, suggesting that maternal periodontitis and the associated bacteria may represent an important systemic stressor for both the mother and fetus (Ruma *et al.*, 2008). Moreover, maternal oral pathogens may reach the developing fetus through haematogenous dissemination (Han *et al.*, 2009). In particular, *Campylobacter rectus* is an exclusively oral gram-negative anaerobe that in experiments has shown the competence to selectively translocate to the fetoplacental unit and operate as a fetal infectious agent eliciting prematurity and growth restriction in animals (Offenbacher *et al.*, 2005; Bobetsis *et al.*, 2007). Furthermore, our human clinical studies have found that maternal *C. rectus* infection in the presence of low serum antibody is associated with high fetal exposure and PTD, as demonstrated by high fetal IgM antibody responses (Madianos *et al.*, 2001). Fetal exposure to oral organisms is associated with higher levels of several proinflammatory mediators in cord blood, including interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α), especially among preterm births. However, the underlying biological mechanisms leading to fetal–placental inflammation and PTD after *C. rectus* exposure still remain to be elucidated.

The World Health Organization defines PTD as birth at <37 completed gestational weeks (1970). Preterm delivery is still the major cause of neonatal mortality and morbidity in the world, associated with low birthweight (<2500 ***g***) and fetal intrauterine growth restriction (MacDorman *et al.*, 2005). Preterm delivery can be initiated by multiple mechanisms including infection, local inflammation, uteroplacental ischemia, hemorrhage, stress and other immunologically mediated processes (Romero *et al.*, 2006). Although precise triggering mechanisms have not been established, the development of a proinflammatory condition is a common pathway that potentiates all multiple PTD risk factors (Romero *et al.*, 1994). Particularly, uterine infections account for 25–40% of preterm births and they are strongly linked to local proinflammatory cytokines, metalloproteinases and prostaglandins. The increased expression of these inflammatory mediators may lead to membrane weakening, early membrane rupture and uterine contraction initiation (Shoji *et al.*, 2007). Such proinflammatory responses are likely to be initiated and/or mediated by the host innate immune system via activation of Toll-like receptors (TLRs), which have a primary role in pathogen recognition and innate immunity initiation (Brikos & O'Neill, 2008). The TLR receptors bind to several microbial components or end-products known as pathogen-associated molecular patterns. After binding and recognition, TLRs are able to trigger an array of signaling pathways that ultimately activate downstream molecules such as nuclear factor-κB and interferon regulatory factor 3 (Uematsu & Akira, 2006), which in turn mediate the expression of several proinflammatory cytokines as demonstrated in several tissues, including the maternal–fetal interface (Koga & Mor, 2008). Regulation of TLR has been suggested to play a critical role in mediating the innate immune response during pregnancy, which in turn has significant implications for the success or failure of pregnancies in both early and late gestation (Patni *et al.*, 2007). To date, about 13 mammalian TLR homologues have been identified and designated, and their expression has been described in the human placenta, being dominantly expressed by trophoblasts (Holmlund *et al.*, 2002; Abrahams *et al.*, 2004; Kumazaki *et al.*, 2004). Notably, trophoblasts have also been proposed to be involved in coordinating the immune response during embryonic implantation, placental development and immunosurveillance (Mor, 2008).

Our overall goal is to better understand the maternal and fetal biological mechanisms leading to PTD in response to *C. rectus* infection. Specifically, we have hypothesized that *C. rectus* induces a placental innate inflammatory response mediated by TLRs. This hypothesis is based on previous studies using *C. rectus* as a model of systemic infection in pregnant mice, in which we have demonstrated: (i) the systemic dissemination of *C. rectus* from distant sites of infection (dorsal subcutaneous chamber and oral cavity) to the placenta (Arce *et al.*, 2010); (ii) increased local

placental inflammatory response confined to the decidua along with placental structural alterations (wider junctional zone) (Offenbacher *et al.*, 2005); (iii) fetal intrauterine growth restriction induction (lighter and shorter fetal pups) (Yeo *et al.*, 2005); (iv) altered gene expression along with downregulation of several imprinted genes (i.e. insulin growth-factor 2) via changes in DNA methylation patterns (hypermethylation) (Bobetsis *et al.*, 2007, 2010); (v) increased trophoblastic TLR4 expression (two-fold) after *C. rectus* oral infection (Arce *et al.*, 2009); and (vi) *in vitro* trophoblastic production of TNF-α and IL-6 in a dose-dependent response to *C. rectus* infection (Arce *et al.*, 2010). Based upon these experimental observations, we believe that the local placental inflammatory response may play a significant role in mediating IUGR. However, it is still unclear whether the activation of TLRs mediates the placental inflammatory responses and IUGR in response to *C. rectus* exposure *in vivo*. The purpose of this investigation was to determine the role of TLR4 in mediating *in vitro* (human trophoblasts) cytokine synthesis following *C. rectus* challenge and to determine whether TLR4-deficient mice would lose the IUGR phenotype in response to *C. rectus* exposure.

METHODS

Mammalian cell lines

The human trophoblast cell line BeWo (ATCC CCL-98) was used for cytokine assays (Pattillo & Gey, 1968). BeWo cells are the first human trophoblastic endocrine cell type to be maintained in continuous culture, initiated from a malignant gestational choriocarcinoma of the fetal placenta. Briefly, BeWo cells were grown in Ham's F12K medium with 2 mM L-glutamine adjusted to contain 10% fetal bovine serum according to ATCC propagation instructions. Cells were grown in T-25 flasks (Corning Life Sciences, Lowell, MA) or onto cover slips placed in six-well plates for the experiments. All cells were grown at 37°C in 10% CO_2.

Bacterial cultures

Campylobacter rectus 314 aliquots were maintained in Wilkins-Chalgren anaerobic broth medium (WC broth; DSMZ, Braunschweig, Germany) containing 10% skim milk at −80°C. The *C. rectus* aliquots were reconstituted on PRAS ETSA plates (Enriched Tryptic Soy Agar from Anaerobe Systems, Morgan Hill, CA). Bacteria were anaerobically grown under 5% CO_2/10% H_2/85% N_2 atmosphere at 37°C for 4–6 days. Bacterial suspensions were prepared from primary cultures at their log phase of growth and resuspended in tissue culture medium without antibiotics (*in vitro* experiments) or in phosphate-buffered saline (PBS) (*in vivo* experiments) to an optical density at 600 nm of 1.00 determined with a CE 2041 UV/VIS spectrophotometer (Cecil Instruments, Cambridge, UK) corresponding to 1×10^9 bacteria ml^{-1}.

In vitro trophoblast infection assays

BeWo cells were grown onto six-well plates until 80–90% confluency. BeWo cell monolayers were also washed three times with cell culture medium without antibiotics before inoculation with bacteria. Bacterial cells were added to obtain a multiplicity of infection of 500 bacteria (MOI_{500})/BeWo cell, after which plates were centrifuged at 250 ***g*** for 5 min, incubated for 12 h at 37°C in 10% CO_2 and washed with PBS. This time-point and MOI were chosen based on previous experiments demonstrating a dose-dependent proinflammatory activity (Arce *et al.*, 2010). All experiments were performed in triplicate and at two independent times.

TLR4 neutralization

Additional infection experiments were performed to evaluate the effect of using a TLR4 neutralizing antibody in the proinflammatory phenotype. Briefly, BeWo cells were treated with 1 or 2 μg anti-human TLR4 antibody (AF1478 goat IgG; R&D Systems, Minneapolis, MN) for 2 h before infection. Then BeWo cell monolayers were washed three times with cell culture medium without antibiotics and were followed by the infection protocol as explained before. The concentration for human TLR4 bioactivity neutralization for this antibody was chosen based on the lowest dose recommended by the manufacturer (1.5 μg ml^{-1}). Additional experiments were also performed to include ultrapure *Escherichia coli* lipopolysaccharide (LPS) (0111:B4 strain; Invivogen, San Diego, CA) using 1 μg per well as a positive control for the production of proinflammatory cytokines.

Cytokine multiplexing assays

Infected cells and non-infected controls were washed three times with PBS to remove non-adhered cells. The quantification of IL-6 and TNF-α in cell supernatants was performed by means of xMAP multiplex cytokine assays. Briefly, cell supernatants were collected after timed infection, centrifuged at 1500 ***g*** for 5 min and then frozen until analysis. Multianalyte kits for human and mouse IL-6 and TNF-α were used following the manufacturer's instructions (Fluorokine MAP Kits, R&D Systems, Minneapolis, MN). Experiments were performed in duplicate with two independent assays.

Congenic TLR4-deficient mouse model of systemic *C. rectus* infection

All procedures were in accordance with the animal welfare guidelines and approved by the University of North Carolina-Chapel Hill Institutional Animal Care and Use Committee. The mouse infection model used was similar to that described before (Yeo *et al.*, 2005). BALB/cAnPt TLR4-deficient ($TLR4^{Lps\text{-}d}$) mice and congenic BALB/cByJ Wild-type (WT) controls were obtained from the Jackson Laboratory (C.C3-$Tlr4^{Lps\text{-}d}$/J stock number 002930 and BALB/cByJ wild-type stock number 001026; The Jackson Laboratory, Bar Harbor, MA). All mice were housed under controlled and standardized conditions with 12-h light–dark cycles. Regular mouse diet and water were provided *ad libitum*. Female mice were enrolled in the experiments at approximately 6 weeks of age and immediately had a steel chamber implanted subcutaneously as previously described (Yeo *et al.*, 2005). After 1 month of healing, the female mice were mated overnight with male mice of the same or different background (six female mice per group). The next morning, female mice were removed from the cages of the male mice and examined for vaginal plugs. If a plug was found, that day was recorded as embryonic day E0.5. At E7.5, pregnant mice received an intra-chamber injection of 100 μl of 10^9 colony-forming units ml^{-1} live *C. rectus* in PBS. Mice were then sacrificed at E16.5 and fetuses (n = 47 from homozygous $TLR4^{Lps\text{-}d-/-}$ infected dams and n = 49 from heterozygous $TLR4^{Lps\text{-}d+/-}$ infected dams) and their respective placental tissues were collected for further analyses (fetal length/weight). For comparison purposes, we included fetal weight and length data from experiments using sham-infected WT controls (n = 143) and *C. rectus*-infected WT mice (n = 37) that were subjected to the same experimental protocol.

Statistical analysis

A minimal sample size of six mice per group was calculated [power $(1 - \beta)$ of >0.90% with alpha-error threshold of $\alpha = 0.05$ based on our previous results on fetal growth restriction after *C. rectus* systemic infection at E16.5 (Arce *et al.*, 2010). Continuous variables were expressed as means and standard errors. The mRNA fold differences between the infected cells (test) and non-infected controls were compared using the unpaired *t*-test. Protein concentration differences were compared using the one-way analysis of variance (ANOVA) test. Mean placental/fetal weight and fetal length values for all groups were compared using the ANOVA (Kruskal–Wallis) test with Dunn's post-hoc comparisons. The frequency of resorptions and litter sizes in all groups were compared by using the chi-square test. The threshold for statistical significance was set at a $P < 0.05$. No corrections were made for multiple comparisons. All analyses were performed using GRAPHPAD software (San Diego, CA).

RESULTS

Neutralizing anti-human TLR4 antibodies impaired the production of proinflammatory cytokines in BeWo cells after *C. rectus* infection in a dose-dependent manner

We evaluated the production of the proinflammatory cytokines IL-6 and TNF-α by multiplex assays in response to *C. rectus* treatment and the effects of anti-TLR4 neutralizing antibodies on cytokine synthesis. As depicted in Fig. 1, there was a dose-dependent suppression of the cytokine production by the addition of anti-TLR4 antibody (TLR4AB)… in BeWo cells. For example, for total IL-6 production in response to MOI_{500} there was a statistically significant decrease to 46.4% when cells were pre-treated with 1 μg TLR4AB (34.0 ± 9.02 pg ml^{-1}), and a further decrease to 17.1% when using 2 μg TLR4AB (12.5 ± 4.18 pg ml^{-1}, $P < 0.05$, ANOVA). A similar trend was also observed for TNF-α, in which total production was decreased to 48.1% when using 1 μg TLR4AB (4.9 ± 1.80 pg ml^{-1}) and to 33.6% when using 2 μg TLRAB (3.4 ±

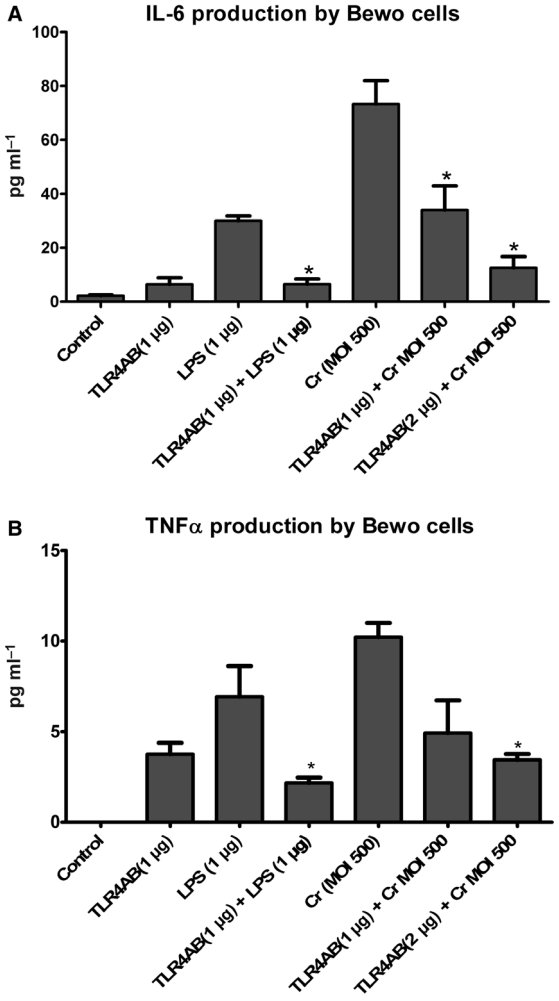

Figure 1 TLR4 neutralization in trophoblastic cells affects cytokine production after *in vitro C. rectus* infection. Box-plots depicting mean ± SEM. BeWo cells were pre-treated with 1ug or 2ug of a TLR4AB showing a dose-response decrease in A) IL-6 and B) TNFα production. Cr = *C. rectus*. TLR4AB = neutralizing antibody. MOI = multiplicity of infection. * indicates statistical significance (P <0.05, ANOVA) when compared to blank controls and highest infection dose [Cr (MOI 500)].

0.32 pg ml^{-1}, $P < 0.05$, ANOVA). We also evaluated the proinflammatory responses to a classical TLR4 agonist (*E. coli* LPS) as a positive control for the experiment, finding a significant decrease for both IL-6 (21.5%) and TNF-α (31%).

TLR4-deficient fetuses are less susceptible to IUGR after maternal *C. rectus* systemic infection

We sought to evaluate the *in vivo* effect of a deficient TLR4 receptor congenic murine strain on IUGR after systemic *C. rectus* exposure. In terms of litter size and frequency of fetal resorptions, there were no statistical differences among all experimental groups ($P > 0.05$, chi-square). This suggests that there were no effects of TLR4 deficiency on implantation. However, differences in fetal weight are shown in Fig. 2. The WT fetuses from sham-infected dams had an average weight of 0.47 ± 0.005 g. When infected with *C. rectus*, WT dams delivered IUGR fetuses that had an average weight of 0.42 ± 0.009 g. However, when TLR4$^{Lps\text{-}d}$ dams were mated with male mice of the same genetic background and then infected with *C. rectus*, the TLR4$^{Lps\text{-}d-/-}$ homozygous fetuses appeared not to be affected by the infection as their average weight of 0.46 ± 0.010 did not statistically differ from the WT control dams ($P > 0.05$, Kruskal–Wallis). Furthermore, when TLR4$^{Lps\text{-}d}$ dams were mated with a male mouse from a different genetic background (WT) and after *C. rectus* infection, the IUGR phenotype of TLR4$^{Lps\text{-}d+/-}$ heterozygous fetuses had an average fetal weight of 0.43 ± 0.008 g and was statistically different from the WT controls ($P < 0.05$, Kruskal–Wallis) and intermediate between WT and homozyogousTLR4$^{Lps\text{-}d-/-}$. A very similar trend was also observed for the fetal length (expressed in cm) as illustrated in Fig. 3. For example, WT controls had an average size of 1.440 ± 0.008 g and when WT dams were infected by *C. rectus*, fetuses were found to be significantly smaller (1.229 ± 0.01 g, $P < 0.05$, Kruskal–Wallis). However, infected homozygous TLR4$^{Lps\text{-}d-/-}$ fetuses were larger than infected WT (1.396 ± 0.019 g) and were not statistically different from WT controls ($P > 0.05$). On the other hand, infected heterozygous TLR4$^{Lps\text{-}d+/-}$ mice showed smaller fetuses (1.251 ± 0.012 g) that were significantly different from fetuses from the WT controls.

DISCUSSION

Preterm delivery is the major cause of neonatal morbidity/mortality in the world, affecting 12.5% of live births in the USA (Abrahams, 2008). Even though the etiology of pregnancy complications remains somewhat elusive, there is strong evidence supporting the association between infections and PTD, mostly mediated by systemic inflammation (Goldenberg *et al.*, 2000; Romero *et al.*, 2007). These observations in humans have been confirmed by a number of animal models demonstrating a PTD phenotype in response to experimental infection with either heat-killed, live

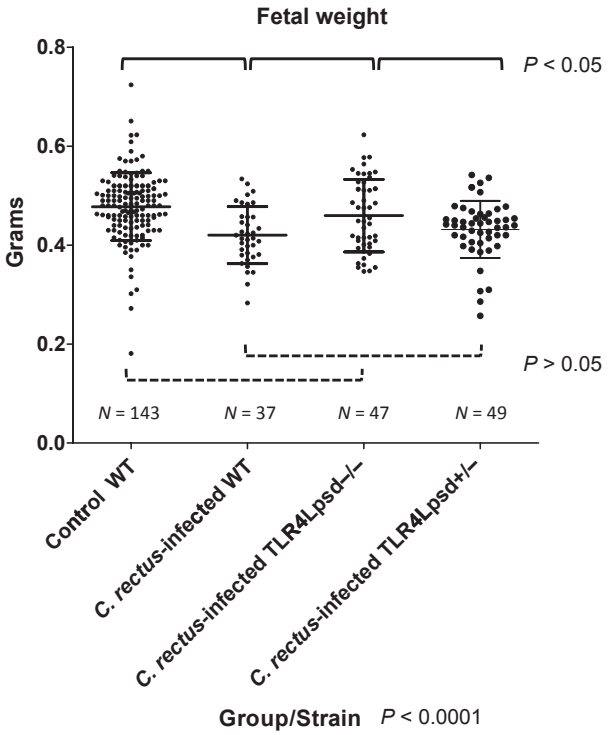

Figure 2 Fetal weight is not significantly affected in Toll-like receptor-4 (TLR4) -deficient fetuses from *Campylobacter rectus*-infected dams. Box-plots depicting average ± standard errors. The overall Kruskal–Wallis test was highly statistically significant ($P < 0.0001$). Dunn's post-hoc multiple comparison tests found statistically significant differences ($P < 0.05$) for Control wild-type (WT) vs. *C. rectus*-infected WT, Control WT vs. *C. rectus*-infected TLR4$^{Lps\text{-}d+/-}$ and *C. rectus*-infected WT vs. *C. rectus*-infected TLR4$^{Lps\text{-}d-/-}$. There were no significant differences ($P > 0.05$) for Control WT vs. *C. rectus*-infected TLR4$^{Lps\text{-}d-/-}$, *C. rectus*-infected WT vs. *C. rectus*-infected TLR4$^{Lps\text{-}d+/-}$ and *C. rectus*-infected TLR4$^{Lps\text{-}d-/-}$ vs. *C. rectus*-infected TLR4$^{Lps\text{-}d+/-}$. Solid lines indicate statistical significance ($P < 0.05$). Dotted lines indicate no statistical significance ($P > 0.05$).

bacteria or isolated bacterial components that are able to trigger an inflammatory cascade at the feto-placental level (Han *et al.*, 2004; Elovitz, 2006).

Toll-like receptors have gained much attention in PTD research since 2000. The TLRs are a family of transmembrane proteins that play a key role in activating the innate immune system in different organs including the fetal maternal interphase (Uematsu & Akira, 2006). In particular, TLR4 has been found to be particularly important because it senses the major gram-negative component – LPS. Lipopolysaccharide, by either systemic, intrauterine, or intra-amniotic delivery, triggers PTD *in vivo* (Elovitz & Mrinalini, 2004; Liu *et al.*, 2007). Furthermore, mice deficient for TLR4 show protection against bacterial and LPS-induced PTD (Elovitz *et al.*, 2003; Wang & Hirsch, 2003). Experimental TLR4 antagonism suppresses proinflammatory responses to bacteria including oral microorganisms *in vivo* (Liu *et al.*, 2007; Adams Waldorf *et al.*, 2008). At the cellular level, trophoblasts are thought to be primary sentinel cells that are involved in microbial clearance preventing microbial translocation of infectious agents from mother to fetus (Levy, 2007). Upon recognition of microbes through TLRs, trophoblasts coordinate recruitment of natural killer cells and neutrophils to the maternal–fetal interface, a key feature in PTD and pre-eclampsia pathogenesis (Abrahams & Mor, 2005). Trophoblastic cells upregulate the secretion of proinflammatory chemokines (IL-8 and monocyte chemoattractant protein-1) and cytokines (IL-1β, IL-6 and TNF-α) following the ligation of TLR4 by bacterial LPS, inducing trophoblastic cells to differentially modulate the maternal immune system both during normal pregnancy and in the presence of an intrauterine infection (Mor, 2008).

A similar proinflammatory phenotype has also been observed in our experiments using the oral periodontal pathogen *Campylobacter rectus*; this motile, gram-negative anaerobe plays a pathogenic role in human periodontitis (Rams *et al.*, 1993; Yokoyama *et al.*, 2008). *Campylobacter rectus* shows a wide array of virulence factors including LPS (Ogura *et al.*, 1995, 1996; Takiguchi *et al.*, 1996). It is part of the Campylobacteraceae family, which has been associated with other diseases showing important proinflammatory mechanistic similarities such as *Campylobacter jejuni* in acute gastroenteritis (Allos, 2001) and *Campylobacter fetus* in sheep and cattle abortion (Guerrant *et al.*, 1978; Macuch & Tanner, 2000; Fujihara *et al.*, 2006). In animal models, *C. jejuni* and *C. fetus* infections result in impaired fetal development and IUGR (O'Sullivan *et al.*, 1988a,b). Our *in vivo* and *in vitro C. rectus* infection experiments in pregnant mice and placental cells have consistently shown an upregulation of maternal inflammatory serum markers following challenge (Yeo *et al.*, 2005), as well as placental translocation. In culture, *C. rectus* is capable of trophoblastic cell invasion. These observations suggest a pathway that involves translocation of *C. rectus* to placental tissue, activation of trophoblastic TLR4 and a consequent placental proinflammatory response that ultimately results in IUGR (Arce *et al.*, 2010).

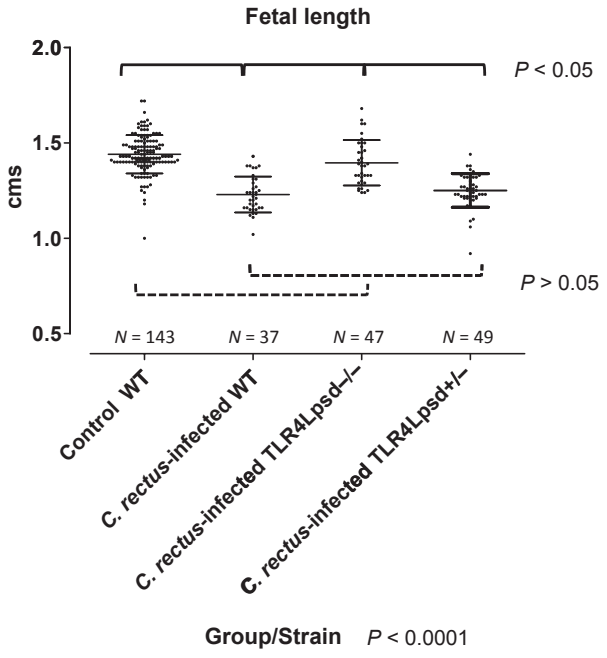

Figure 3 Fetal length is unaffected in Toll-like receptor-4 (TLR4) - deficient fetuses from *Campylobacter rectus*-infected dams. Boxplots depicting average ± SEM. The overall Kruskal–Wallis test was highly statistically significant ($P < 0.0001$). Dunn's post-hoc multiple comparison tests found statistically significant differences ($P < 0.05$) for Control wild-type (WT) vs. *C. rectus*-infected WT, Control WT vs. *C. rectus*-infected TLR4$^{Lps\text{-}d+/-}$, *C. rectus*-infected WT vs. *C. rectus*-infected TLR4$^{Lps\text{-}d-/-}$ and for *C. rectus*-infected TLR4$^{Lps\text{-}d-/-}$ vs. *C. rectus*-infected TLR4$^{Lps\text{-}d+/-}$. There were no significant differences for Control WT vs. *C. rectus*-infected TLR4$^{Lps\text{-}d-/-}$ and *C. rectus*-infected WT vs. *C. rectus*-infected TLR4$^{Lps\text{-}d+/-}$. Solid lines indicate statistical significance ($P < 0.05$). Dotted lines indicate no statistical significance ($P > 0.05$).

We previously reported a dose-dependent response for human IL-6 and TNF-α production at the protein level in human BeWo trophoblasts in response to *C. rectus* infection *in vitro* (Arce *et al.*, 2010). In this report we provide new data that demonstrate that the antagonism of TLR4 by means of a neutralizing TLR4 antibody (TLR4AB) impairs the activation of inflammatory responses (Fig. 1). When BeWo cells were pretreated with two different doses (1 or 2 μg) of TLR4AB for 2 h before live bacterial exposure, a statistically significant dose-dependent inhibition in the level of IL-6 was observed (Fig 1A, $P < 0.05$, ANOVA for both doses when compared with the *C. rectus* MOI_{500}). A similar trend was observed for TNF-α production (Fig. 1B, $P < 0.05$, ANOVA for 2 μg dose when compared with the *C. rectus* MOI_{500}). The ligation of TLR4 by the antagonist also induced some degree of cytokine activation that was not statistically significant when compared with the blank control ($P > 0.05$, ANOVA), but may indicate a slight partial agonist activity. Collectively, these results strongly suggest a TLR4-dependent activity of BeWo cells in response to *C. rectus* infection. Experimental TLR4 antagonism has also demonstrated significant effects on a PTD phenotype in different animal models. For example, TLR4 receptors have been shown to mediate the murine placental inflammatory response and fetal death to *Fusobacterium nucleatum*, another oral periodontal bacterial species associated with PTD (Liu *et al.*, 2007). Moreover, the selective antagonism of TLR4 inhibits inflammation and preterm uterine contractility in a nonhuman intra-amniotic LPS model in Rhesus monkeys (Adams Waldorf *et al.*, 2008).

Lastly, we aimed to evaluate our current murine model of systemic bacterial exposure of *C. rectus* in pregnant mice on a congenic TLR4-mutant mouse strain on a BALB/c background. As reported before, a remote subcutaneous maternal *C. rectus* infection increases fetal growth restriction in BALB/c mice (Offenbacher *et al.*, 2005; Yeo *et al.*, 2005). As shown in Figs 2 and 3, when WT dams were infected with *C. rectus*, there was a statistically significant decrease in fetal weight (12.5%) and length reduction (14.7%) ($P < 0.05$, Kruskal–Wallis). However, in the absence of functional TLR4 in homozygous fetuses, no IUGR was detected as TLR4$^{Lps\text{-}d-/-}$ fetuses were not statistically different from sham-infected WT control mice for both weight and length ($P > 0.05$, Dunn's). Furthermore, when fetuses carried only one allele with a functional TLR4 in the heterozygous TLR4$^{Lps\text{-}d+/-}$ group the IUGR phenotype was again observed as these fetuses were significantly different from WT control mice ($P < 0.05$, Dunn's) and comparable to *C. rectus*-infected WT dams ($P > 0.05$, Dunn's). In our interpretation, the observed phenotype in our experiments completely supported our hypothesis that TLR4 receptors are mediating IUGR after systemic *C. rectus* infection in mice. This is also in agreement with Liu *et al.* (2007), who reported that the fetal death rate was significantly reduced in TLR4-deficient mice on a TLR4 knockout strain following *F. nucleatum* infection.

In conclusion, TLR4 neutralization leads to a significant reduction in trophoblastic proinflammatory activity that can be observed *in vitro*. Moreover, the absence of functional TLR4 in mice seems to lead to

decreased susceptibility to *C. rectus* infection (and IUGR) *in vivo*. Collectively, these results suggest that the placental proinflammatory responses are mediated by TLR4 during low-birthweight/PTD pathogenesis. We also speculate that TLR4-antagonistic therapies may be used to specifically block infection-associated inflammation during pregnancy as proposed by others (Liu *et al.*, 2007; Abrahams, 2008). As a therapeutic target, TLR4 may provide future promise and future studies may bring new anti-inflammatory agents for the treatment or prevention of bacterially induced PTD.

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REFERENCES

Abrahams, V.M. (2008) Antagonizing toll-like receptors to prevent preterm labor. *Reprod Sci* **15**: 108–109.

Abrahams, V.M. and Mor, G. (2005) Toll-like receptors and their role in the trophoblast. *Placenta* **26**: 540–547.

Abrahams, V.M., Bole-Aldo, P., Kim, Y.M. *et al.* (2004) Divergent trophoblast responses to bacterial products mediated by TLRs. *J Immunol* **173**: 4286–4296.

Adams Waldorf, K.M., Persing, D., Novy, M.J., Sadowsky, D.W. and Gravett, M.G. (2008) Pretreatment with toll-like receptor 4 antagonist inhibits lipopolysaccharide-induced preterm uterine contractility, cytokines, and prostaglandins in rhesus monkeys. *Reprod Sci* **15**: 121–127.

Albandar, J.M. (2002) Global risk factors and risk indicators for periodontal diseases. *Periodontol 2000* **29**: 177–206.

Allos, B.M. (2001) *Campylobacter jejuni* infections: update on emerging issues and trends. *Clin Infect Dis* **32**: 1201–1206.

Arce, R.M., Barros, S.P., Wacker, B., Peters, B., Moss, K. and Offenbacher, S. (2009) Increased TLR4 expression in murine placentas after oral infection with periodontal pathogens. *Placenta* **30**: 156–162.

Arce, R.M., Diaz, P.I., Barros, S.P. *et al.* (2010) Characterization of the invasive and inflammatory traits of oral *Campylobacter rectus* in a murine model of fetoplacental growth restriction and in trophoblast cultures. *J Reprod Immunol* **84**: 145–153.

Beck, J.D. and Offenbacher, S. (2005) Systemic effects of periodontitis: epidemiology of periodontal disease and cardiovascular disease. *J Periodontol* **76**: 2089–2100.

Bobetsis, Y.A., Barros, S.P., Lin, D.M. *et al.* (2007) Bacterial infection promotes DNA hypermethylation. *J Dent Res* **86**: 169–174.

Bobetsis, Y.A., Barros, S.P., Lin, D.M., Arce, R.M. and Offenbacher, S. (2010) Altered gene expression in murine placentas in an infection-induced intrauterine growth restriction model: a microarray analysis. *J Reprod Immunol* **85**: 140–148.

Brikos, C. and O'Neill, L.A. (2008) Signalling of toll-like receptors. *Handb Exp Pharmacol* **183**: 21–50.

Elovitz, M.A. (2006) Anti-inflammatory interventions in pregnancy: now and the future. *Semin Fetal Neonatal Med* **11**: 327–332.

Elovitz, M.A. and Mrinalini, C. (2004) Animal models of preterm birth. *Trends Endocrinol Metab* **15**: 479–487.

Elovitz, M.A., Wang, Z., Chien, E.K., Rychlik, D.F. and Phillippe, M. (2003) A new model for inflammation-induced preterm birth: the role of platelet-activating factor and Toll-like receptor-4. *Am J Pathol* **163**: 2103–2111.

Fujihara, N., Takakura, S., Saito, T., Iinuma, Y. and Ichiyama, S. (2006) A case of perinatal sepsis by *Campylobacter fetus* subsp. fetus infection successfully treated with carbapenem—case report and literature review. *J Infect* **53**: e199–e202.

Goldenberg, R.L., Hauth, J.C. and Andrews, W.W. (2000) Intrauterine infection and preterm delivery. *N Engl J Med* **342**: 1500–1507.

Guerrant, R.L., Lahita, R.G., Winn, W.C. Jr and Roberts, R.B. (1978) Campylobacteriosis in man: pathogenic mechanisms and review of 91 bloodstream infections. *Am J Med* **65**: 584–592.

Han, Y.W., Redline, R.W., Li, M., Yin, L., Hill, G.B. and McCormick, T.S. (2004) *Fusobacterium nucleatum* induces premature and term stillbirths in pregnant mice: implication of oral bacteria in preterm birth. *Infect Immun* **72**: 2272–2279.

Han, Y.W., Shen, T., Chung, P., Buhimschi, I.A. and Buhimschi, C.S. (2009) Uncultivated bacteria as etiologic agents of intra-amniotic inflammation leading to preterm birth. *J Clin Microbiol* **47**: 38–47.

Holmlund, U., Cebers, G., Dahlfors, A.R. *et al.* (2002) Expression and regulation of the pattern recognition receptors Toll-like receptor-2 and Toll-like receptor-4 in the human placenta. *Immunology* **107**: 145–151.

Koga, K. and Mor, G. (2008) Expression and function of toll-like receptors at the maternal–fetal interface. *Reprod Sci* **15**: 231–242.

Kumazaki, K., Nakayama, M., Yanagihara, I., Suehara, N. and Wada, Y. (2004) Immunohistochemical distribution of Toll-like receptor 4 in term and preterm human placentas from normal and complicated pregnancy including chorioamnionitis. *Hum Pathol* **35**: 47–54.

Levy, O. (2007) Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat Rev Immunol* **7**: 379–390.

Liu, H., Redline, R.W. and Han, Y.W. (2007) *Fusobacterium nucleatum* induces fetal death in mice via stimulation of TLR4-mediated placental inflammatory response. *J Immunol* **179**: 2501–2508.

MacDorman, M.F., Martin, J.A., Mathews, T.J., Hoyert, D.L. and Ventura, S.J. (2005) Explaining the 2001–2002 infant mortality increase in the United States: data from the linked birth/infant death data set. *Int J Health Serv* **35**: 415–442.

Macuch, P.J. and Tanner, A.C. (2000) *Campylobacter* species in health, gingivitis, and periodontitis. *J Dent Res* **79**: 785–792.

Madianos, P.N., Lieff, S., Murtha, A.P. *et al.* (2001) Maternal periodontitis and prematurity. Part II: maternal infection and fetal exposure. *Ann Periodontol* **6**: 175–182.

Mor, G. (2008) Inflammation and pregnancy: the role of toll-like receptors in trophoblast-immune interaction. *Ann N Y Acad Sci* **1127**: 121–128.

Offenbacher, S., Riche, E.L., Barros, S.P., Bobetsis, Y.A., Lin, D. and Beck, J.D. (2005) Effects of maternal *Campylobacter rectus* infection on murine placenta, fetal and neonatal survival, and brain development. *J Periodontol* **76**: 2133–2143.

Ogura, N., Shibata, Y., Matsuda, U. *et al.* (1995) Effect of *Campylobacter rectus* LPS on plasminogen activator-plasmin system in human gingival fibroblast cells. *J Periodontal Res* **30**: 132–140.

Ogura, N., Matsuda, U., Tanaka, F., Shibata, Y., Takiguchi, H. and Abiko, Y. (1996) *In vitro* senescence enhances IL-6 production in human gingival fibroblasts induced by lipopolysaccharide from *Campylobacter rectus*. *Mech Ageing Dev* **87**: 47–59.

O'Sullivan, A.M., Dore, C.J., Boyle, S., Coid, C.R. and Johnson, A.P. (1988a) The effect of campylobacter lipopolysaccharide on fetal development in the mouse. *J Med Microbiol* **26**: 101–105.

O'Sullivan, A.M., Dore, C.J. and Coid, C.R. (1988b) Campylobacters and impaired fetal development in mice. *J Med Microbiol* **25**: 7–12.

Patni, S., Flynn, P., Wynen, L.P. *et al.* (2007) An introduction to Toll-like receptors and their possible role in the initiation of labour. *BJOG* **114**: 1326–1334.

Pattillo, R.A. and Gey, G.O. (1968) The establishment of a cell line of human hormone-synthesizing trophoblastic cells *in vitro*. *Cancer Res* **28**: 1231–1236.

Rams, T.E., Feik, D. and Slots, J. (1993) *Campylobacter rectus* in human periodontitis. *Oral Microbiol Immunol* **8**: 230–235.

Romero, R., Mazor, M., Munoz, H., Gomez, R., Galasso, M. and Sherer, D.M. (1994) The preterm labor syndrome. *Ann N Y Acad Sci* **734**: 414–429.

Romero, R., Espinoza, J., Kusanovic, J.P. *et al.* (2006) The preterm parturition syndrome. *BJOG* **113**(Suppl. 3): 17–42.

Romero, R., Espinoza, J., Goncalves, L.F., Kusanovic, J.P., Friel, L. and Hassan, S. (2007) The role of inflammation and infection in preterm birth. *Semin Reprod Med* **25**: 21–39.

Ruma, M., Boggess, K., Moss, K. *et al.* (2008) Maternal periodontal disease, systemic inflammation, and risk for preeclampsia. *Am J Obstet Gynecol* **198**: 389–395.

Shoji, T., Yoshida, S., Mitsunari, M. *et al.* (2007) Involvement of p38 MAP kinase in lipopolysaccharide-induced production of pro- and anti-inflammatory cytokines and prostaglandin E_2 in human choriodecidua. *J Reprod Immunol* **75**: 82–90.

Takiguchi, H., Yamaguchi, M., Mochizuki, K. and Abiko, Y. (1996) Effect of *in vitro* aging on *Campylobacter rectus* lipopolysaccharide-stimulated PGE_2 release from human gingival fibroblasts. *Oral Dis* **2**: 202–209.

Uematsu, S. and Akira, S. (2006) Toll-like receptors and innate immunity. *J Mol Med* **84**: 712–725.

Wang, H. and Hirsch, E. (2003) Bacterially-induced preterm labor and regulation of prostaglandin-metabolizing enzyme expression in mice: the role of toll-like receptor 4. *Biol Reprod* **69**: 1957–1963.

WHO (1970) The prevention of perinatal mortality and morbidity. Report of a WHO Expert Committee. *World Health Organ Tech Rep Ser* **457**: 1–60.

Yeo, A., Smith, M.A., Lin, D. *et al.* (2005) *Campylobacter rectus* mediates growth restriction in pregnant mice. *J Periodontol* **76**: 551–557.

Yokoyama, M., Hinode, D., Yoshioka, M. *et al.* (2008) Relationship between *Campylobacter rectus* and periodontal status during pregnancy. *Oral Microbiol Immunol* **23**: 55–59.