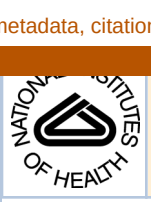

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TLR4 mediate intrauterine growth restriction after systemic *C. rectus* infection in mice

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Summary

Campylobacter rectus is associated with fetal exposure and low-birth weight in humans. *C. rectus* also invades placental tissues and induces fetal intrauterine growth-restriction (IUGR) in mice, along with Toll-like receptors (TLR4) overexpression, suggesting that TLR4 may mediate placental immunity and IUGR in mice. To test this hypothesis we examined the effect of *in vitro*TLR4 neutralization in 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*; pro-inflammatory cytokine production was assessed by cytokine multiplexing assays. Neutralizing TLR4 antibodies significantly impaired the production of pro-inflammatory 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 to sham WT controls($P<0.05$, ANOVA). However, infected $TLR4^{Lps\text{-}d-/-}$ mice did not show statistically significant differences in fetal weight and length when compared to WT controls($P>0.05$). Furthermore, heterozygous $TLR4^{Lps\text{-}d\ -/+}$fetuses showed IUGR phenotype rescue. We concluded 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 birth weight/preterm delivery pathogenesis.

Keywords

periodontitis; preterm delivery; animal models; mice; fetal growth retardation; Campylobacter rectus; Toll-like receptors

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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 and Offenbacher, 2005). Periodontitis has also been associated with an increased risk for preterm delivery (PTD) and preeclampsia 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 hematogenous dissemination (Han *et al.*, 2009). In particular, *Campylobacter rectus* is an exclusively oral Gram negative anaerobe that has experimentally shown the competence to selectively translocate to the fetoplacental unit and operate as a fetal infectious agent eliciting prematurity and growth restriction in animals (Bobetsis *et al.*, 2007;Offenbacher *et al.*, 2005). Furthermore, our studies have found that maternal *C. rectus* infection in the presence of low serum antibody is associated with high fetal exposure and preterm delivery, as demonstrated by high fetal IgM antibody responses (Madianos *et al.*, 2001). Fetal exposure to oral organisms is associated with higher levels of several pro-inflammatory mediators in cord blood, including IL-1β, IL-6 and TNFα, especially among preterm births. However, the underlying biological mechanisms leading to fetal-placental inflammation and preterm delivery after *C. rectus* exposure still remain to be elucidated.

PTD is defined by the World Health Organization as birth at less than 37 completed gestational weeks(1970). PTD is still the major cause of neonatal mortality and morbidity in the world, associated with low birth weight (<2500 grams) and fetal intrauterine growth-restriction(IUGR) (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 centralizes 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 and O'Neill, 2008). TLRs receptors bind to several microbial components or end-products known as pathogen-associated molecular patterns (PAMPs). After binding and recognition, TLRs are able to trigger an array of signaling pathways that ultimately activate downstream molecules such as nuclear factor kB (NF-kB) and interferon regulatory factor 3 (IRF-3)(Uematsu and Akira, 2006), which in turn mediate the expression of several proinflammatory cytokines as demonstrated in several tissues, including the maternal-fetal interface(Koga and Mor, 2008). TLR regulation 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 Toll-like receptors homologues have been identified and designated, and their expression has been described in the human placenta, being dominantly expressed by trophoblasts (Abrahams *et al.*, 2004; Holmlund *et al.*, 2002; Kumazaki *et al.*, 2004). Notably,

trophoblasts have also been proposed to be involved in coordinating the immune response during both embryonic implantation, placental development and immunosurveillance (Mor, 2008).

Our overall goal is to better understand the maternal and fetal biological mechanisms leading to preterm delivery in response to *C. rectus* infection. Specifically, we have hypothesized that *C. rectus* induces a placental innate inflammatory response mediated by Toll-like receptors (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: 1) the systemic dissemination of *C. rectus* from distant sites of infection (dorsal subcutaneous chamber and oral cavity) to the placenta(Arce *et al.*, 2010); 2) Increased local placental inflammatory response confined to the decidua along with placental structural alterations (wider junctional zone)(Offenbacher *et al.*, 2005); 3) Fetal intrauterine growth restriction induction (lighter and shorter fetal pups)(Yeo *et al.*, 2005); 4) Altered gene expression along with down-regulation of several imprinted genes (i.e. Insulin growth-factor 2) via changes in DNA methylation patterns (hypermethylation) (Bobetsis *et al.*, 2007; Bobetsis *et al.*, 2010); 5)increased trophoblastic TLR-4 expression (2-fold) after *C. rectus* oral infection(Arce *et al.*, 2009) and 6) *in vitro* trophoblastic production of TNFα 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 TLRs activation mediate 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 TLR-4 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 and 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 (FBS) according to ATCC propagation instructions. Cells were grown in T-25 flasks (Corning, Life Sciences, MA) or onto cover slips placed in 6-well plates for the experiments. All cells were grown at 37°C in 10% CO_2.

Bacterial cultures

C. rectus 314 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). 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 PBS (*in vivo* experiments) to an optical density of 1.00 (600 nm) determined by spectrophotometry (Cecil Instruments, Cambridge, UK) corresponding to 1×10^9 bacteria/ml.

In vitro trophoblast infection assays

BeWo cells were grown onto 6-well plates until 80–90% confluency. BeWo cell monolayers were also washed 3 times with cell culture medium without antibiotics prior to inoculation with bacteria. Bacterial cells were added to obtain a multiplicity of infection (MOIs) of 500 bacteria/BeWo cell, after which plates were centrifuged at 250 ×*g* for 5 min, incubated for 12h 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 pro-inflammatory activity (Arce *et al.*, 2010). All experiments were done in triplicates and in two independent times.

TLR 4 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 2ug of anti-human TLR4 antibody (AF1478 goat IgG, R&D systems, Minneapolis MN) for 2 hours before infection. Then BeWo cell monolayers were washed 3 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). Additional experiments were also performed to include ultrapure *E. coli* LPS (0111:B4 strain, Invivogen, San Diego, CA) using 1 μg/well as a positive control for the production of proinflammatory cytokines.

Cytokine multiplexing assays

infected cells and non-infected controls were washed 3 times with PBS to remove non-adhered cells. The quantification of IL-6 and TNFα in cell supernatants was performed by means of xMAP multiplexing 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 TNFα were used following the manufacturer's instructions (Fluorokine MAP Kits, R&D systems, Minneapolis, MN). Experiments were performed in duplicates and 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-hour light-dark cycles. Regular mouse diet and water were provided *ad libitum*. Females 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 one month of healing, females were mated overnight with males of the same or different background (6 females per group). The next morning, females were removed from the male cages 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 CFU/mL 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 6 mice per group was calculated [power ($1-\beta$) 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 at E16.5 (Arce *et al.*, 2010). Continuous variables were expressed as means and standard errors. 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 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-value less than 0.05. No corrections were made for multiple comparisons. All analyses were performed using GraphPad software (San Diego California USA).

Results

Neutralizing anti-human TLR 4 antibodies impaired the production of pro-inflammatory cytokines in BeWo cells after *C. rectus* infection in a dose-dependent manner

We evaluated the production of the pro-inflammatory cytokines IL-6 and TNFα by multiplexing assays in response to *C. rectus* treatment and the effects of anti- TLR4 neutralizing antibodies on cytokine synthesis. As depicted in Figure 1, there was a dose-dependent suppression of the cytokine production by the addition of anti-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 1ug TLR4AB (34.0±9.02 pg/mL), and a further decrease to 17.1% was evident when using 2ug of TLR4AB (12.5±4.18, P<0.05, Anova). A similar trend was also observed for TNFα, in which total production was decreased to 48.1% when using 1ug TLR4AB (4.9±1.80) and to 33.6% when using 2ug TLRAB (3.4±0.32, P<0.05, Anova). We also evaluated the pro-inflammatory 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 TNFα (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 there were no effects of TLR4 deficiency on implantation. However, differences in fetal weight are shown in Figure 2. WT fetuses from sham-infected dams had an average weight of 0.47±0.005 grams. When infected with *C. rectus*, WT dams delivered IUGR fetuses that had an average weight of 0.42±0.009. However, when $TLR4^{lps\text{-}d}$dams were mated with males 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 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 was statistically different from the WT controls (P<0.05, *Kruskal-wallis*) and intermediate between WT and homozygous$TLR4^{lps\text{-}d-/-}$. A very similar trend was also observed for the fetal length (expressed in cms) as illustrated in Figure 3. For example, WT controls had an average size of 1.440±0.008 and when WT dams were infected by *C. rectus* fetuses were found to be significantly smaller (1.229±0.01, P<0.05, *Kruskal-wallis*). However, infected homozygous $TLR4^{lps\text{-}d-/-}$ fetuses were larger than infected WT (1.396±0.019) 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) 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 the United States (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 heat-killed, live bacteria or isolated bacterial components that are able to trigger an inflammatory cascade at the fetoplacental level (Elovitz, 2006; Han *et al.*, 2004).

Toll-like receptors (TLRs) have gained a lot of attention in PTD research in the last decade. 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 and Akira, 2006). In particular, TLR receptors type 4 (TLR4) has been found to be particularly important because TLR4 senses the major Gram negative component: lipopolysaccharide (LPS). LPS delivered systemically, intrauterine or intra-amniotically triggers PTD *in vivo*(Elovitz and Mrinalini, 2004; Liu *et al.*, 2007). Furthermore, mice deficient for TLR4 show protection against bacterial and LPS-induced PTD (Wang and Hirsch, 2003; Elovitz *et al.*, 2003). Experimental TLR4 antagonism suppresses pro-inflammatory responses to bacteria including oral microorganisms *in vivo*(Liu *et al.*, 2007;ms 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 NK cells and neutrophils recruitment to the maternal-fetal interface, a key feature in PTD and pre-eclampsia pathogenesis (Abrahams and Mor, 2005). Trophoblastic cells upregulate the secretion of pro-inflammatory chemokines (IL-8 and MCP-1) and cytokines (IL1β, IL6 and TNFα) following the ligation of TLR-4 by bacterial LPS, exerting 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. C. rectus* is an orally Gram negative anaerobe and motile bacterium that plays a pathogenic role in human periodontitis (Rams *et al.*, 1993; Yokoyama *et al.*, 2008). *C. rectus* shows a wide array of virulence factors including LPS (Ogura *et al.*, 1995; Ogura *et al.*, 1996; Takiguchi *et al.*, 1996). *C. rectus* is part of the Campylobacteraceae family which has been associated with other diseases showing important pro-inflammatory mechanistic similarities such as *Campylobacter jejuni* in acute gastroenteritis (Allos, 2001) and *Campylobacter fetus* in sheep and cattle abortion (Guerrant *et al.*, 1978; Macuch and Tanner, 2000; Fujihara *et al.*, 2006). In animal models, *C. jejuni* and *C. fetus* infections result in impaired fetal development and intrauterine growth restriction (O'Sullivan *et al.*, 1988a; O'Sullivan *et al.*, 1988b). Noteworthy, 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 consequent placental pro-inflammatory response that ultimately result in IUGR (Arce *et al.*, 2010).

We previously reported a dose-dependent response for human IL-6 and TNFα production at the protein levels in human BeWo trophoblasts in response to *C. rectus in vitro* infection (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 pre-treated with 2 different doses (1 or 2 ug) of TLR4AB for 2 hours 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 to *Cr* MOI500). A similar trend was observed for TNFα production (Fig. 1b, $P<0.05$, Anova for 2ug dose when compared to *Cr* MOI500). The ligation of TLR4 by the antagonist also induced some degree of cytokine activation that was not statistically significant when compared to 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. TLR4 experimental 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 (ms 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 (Yeo *et al.*, 2005; Offenbacher *et al.*, 2005). As shown in Figures 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 controls for both weight and length($P>0.05$, Dunn's). Furthermore, when fetuses carried only 1 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 controls ($P<0.05$, Dunn's) and comparable to *C. rectus*-infected WT dams ($P>0.05$, Dunn's). To 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 (Liu *et al.*, 2007), who reported that the fetal death rate was significantly reduced in TLR4-deficient mice on a TLR4 knock/out strain following *F. nucleatum* infection.

In conclusion, TLR4 neutralization leads to a significant reduction in trophoblastic pro-inflammatory activity can be observed *in vitro*. Moreover, in the absence of a functional TLR4 in mice seems to lead to decreased susceptibility to *C. rectus* infection (IUGR) *in vivo*. Collectively, these results suggest that the placental proinflammatory responses are mediated by TLR4 during low birth weight/preterm delivery pathogenesis. We also speculate that TLR4-antagonistic therapies may be used to specifically block infection-associated inflammation during pregnancy as proposed by others (Abrahams, 2008; Liu *et al.*, 2007). TLR4 as a therapeutic target may provide new future studies that could have promise to bring new anti-inflammatory agents for the treatment or prevention of bacterial-induced, preterm delivery.

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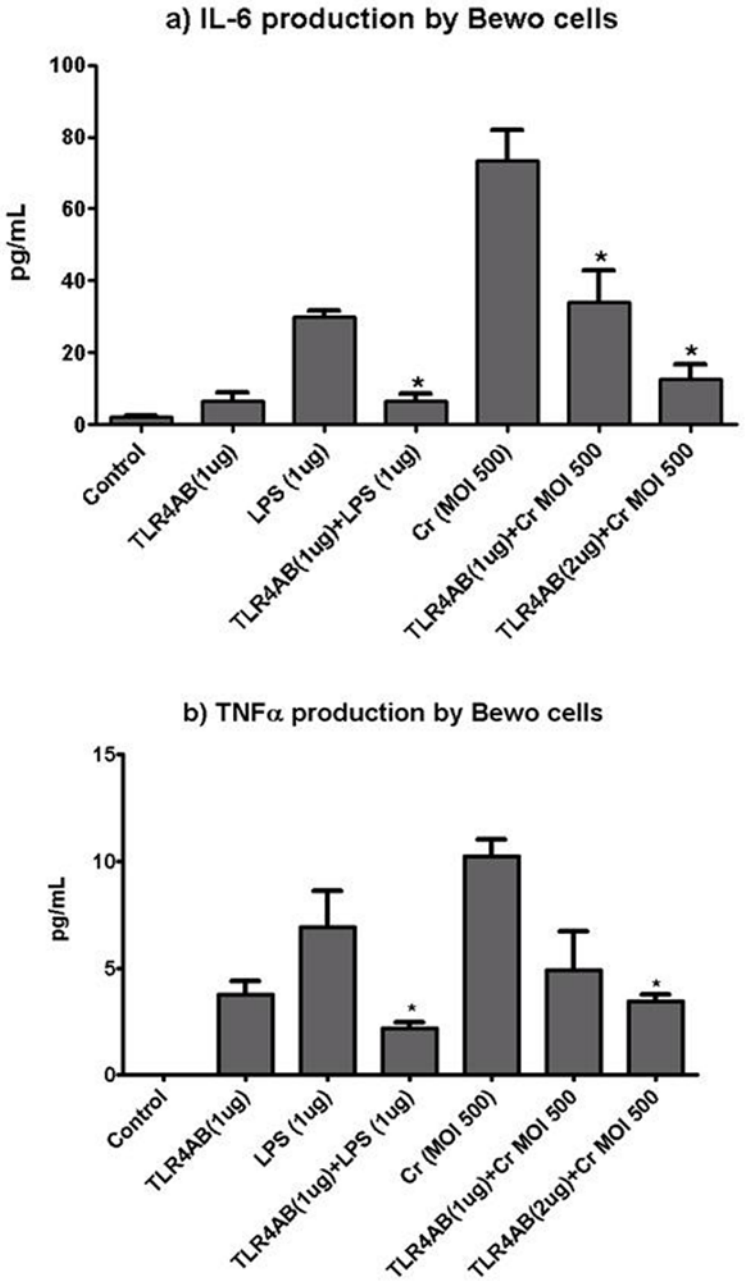

Figure 1. TLR4 neutralization in trophoblastic cells affects cytokine production after in vitro *C. rectus* infection

BeWo cells were pre-treated with 1ug or 2ug of a TLR4AB showing a dose-response decrease in IL-6 and TNFα production. Cr=*C. rectus*. * P<0.05

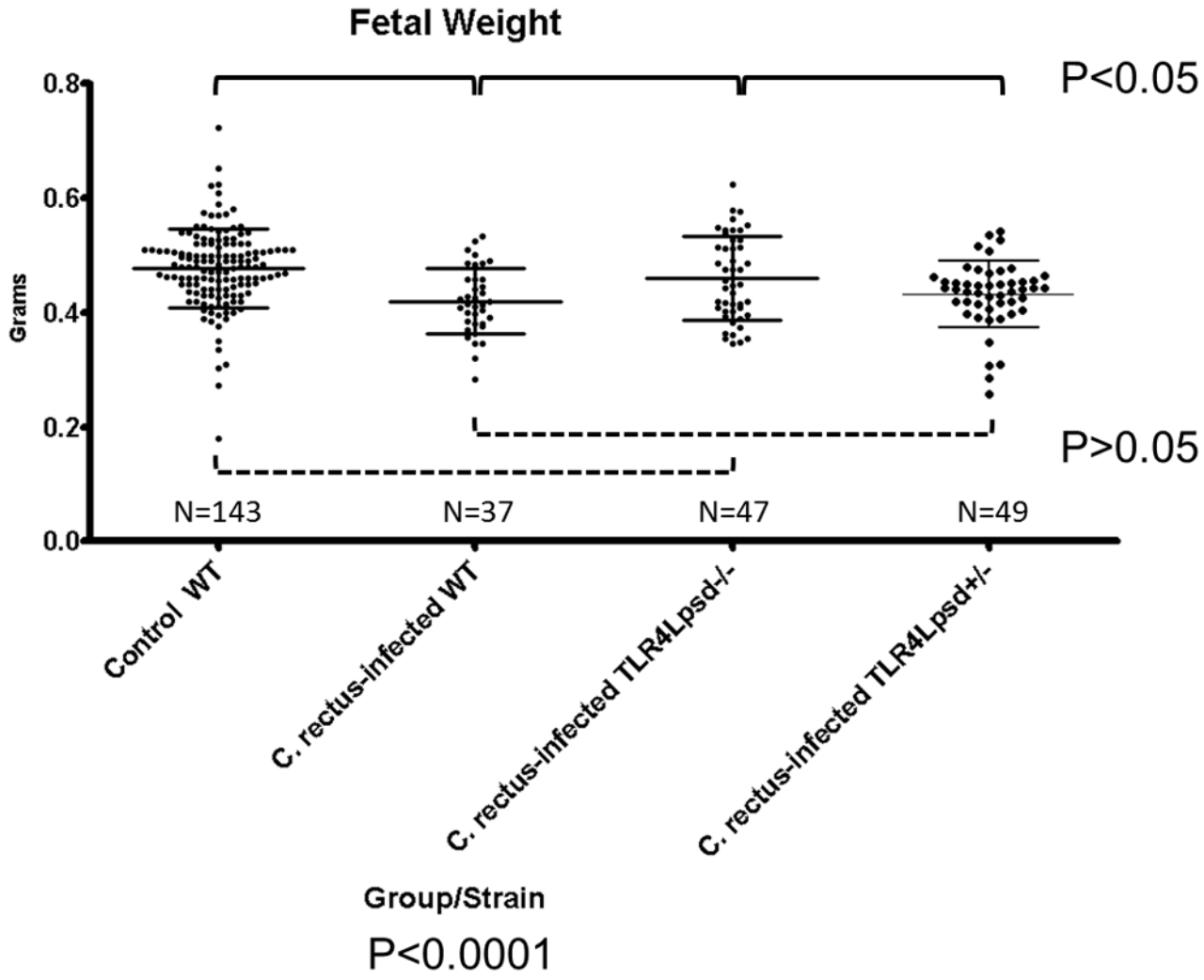

Figure 2. Fetal weight is not significantly affected in TLR4-deficient fetuses from *C. rectus*-infected dams

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 WT vs. *C. rectus*-infected WT, Control WT vs. *C. rectus*-infected TLR4$^{Lpsd+/-}$ and *C. rectus*-infected WT vs. C. rectus-infected TLR4$^{Lpsd-/-}$. There were not significant differences (P>0.05) for Control WT vs. *C. rectus*-infected TLR4$^{Lpsd-/-}$, *C. rectus*-infected WT vs. *C. rectus*-infected TLR4$^{Lpsd+/-}$ and *C. rectus*-infected TLR4$^{Lpsd-/-}$ vs. *C. rectus*-infected TLR4$^{Lpsd+/-}$. Solid lines indicate statistical significance (P<0.05). Dotted lines indicate no statistical significance (P>0.05).

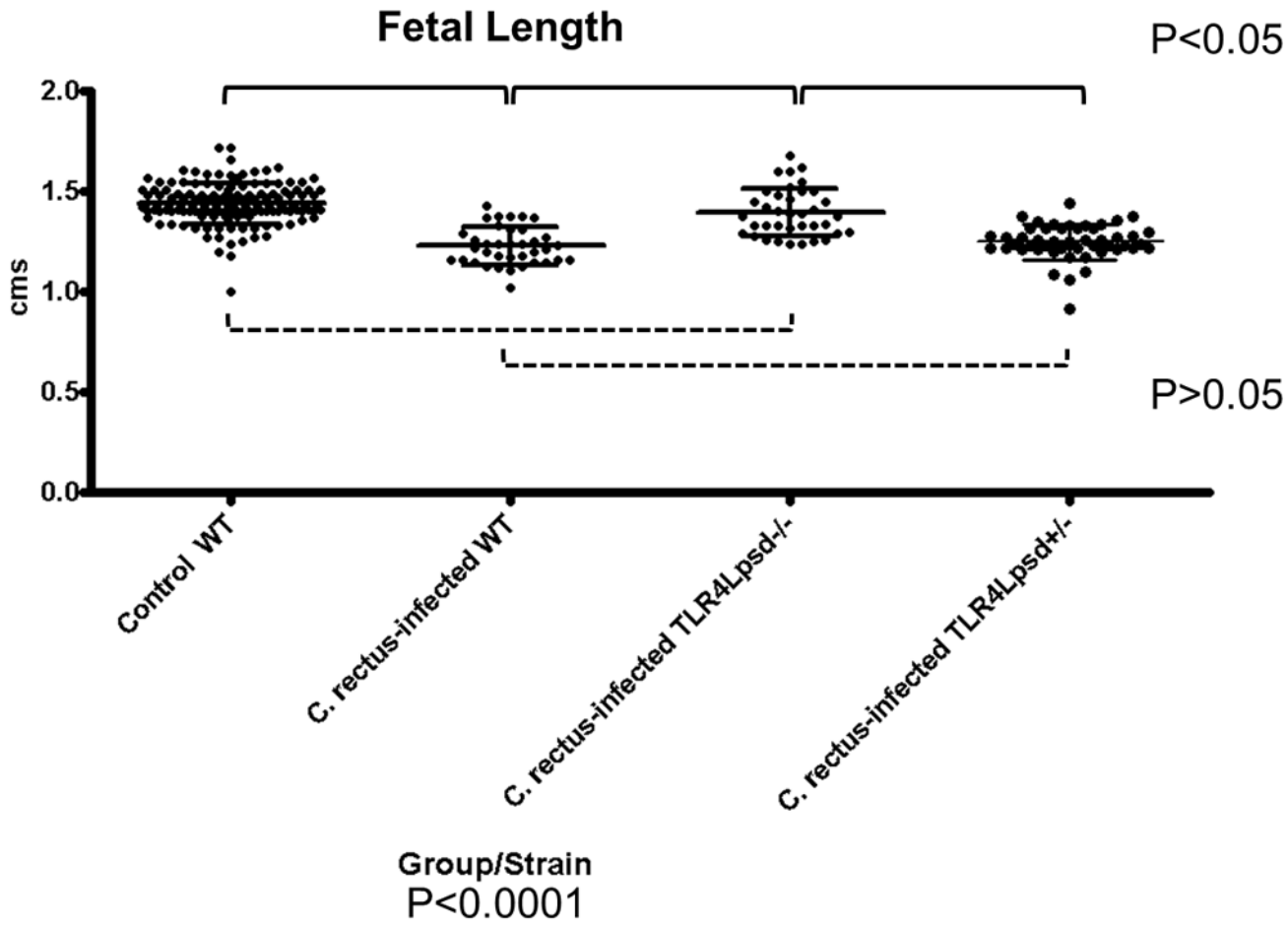

Figure 3. Fetal length is unaffected in TLR4-deficient fetuses from *C. rectus*-infected dams

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 WT vs. *C. rectus*-infected WT, Control WT vs. *C. rectus*-infected TLR4$^{Lpsd+/-}$, *C. rectus*-infected WT vs. *C. rectus*-infected TLR4$^{Lpsd-/-}$ and for *C. rectus*-infected TLR4$^{Lpsd-/-}$ vs. *C. rectus*-infected TLR4$^{Lpsd+/-}$. There were no significant differences for Control WT vs. *C. rectus*-infected TLR4$^{Lpsd-/-}$ and *C. rectus*-infected WT vs. *C. rectus*-infected TLR4$^{Lpsd+/-}$. Solid lines indicate statistical significance (P<0.05). Dotted lines indicate no statistical significance(P>0.05).