Periodontal disease and Preterm Delivery:

Influences of *Campylobacter rectus* Infection on Placental Innate Immunity

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Abstract

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Periodontal disease and Preterm Delivery: Influences of *Campylobacter rectus* Infection on Placental Innate Immunity
(Under the direction of Dr. Steven Offenbacher)

Preterm delivery (PTD) is the major cause of neonatal mortality/ morbidity in the world. PTD pathogenesis can be initiated by multiple mechanisms; however inflammation is the most crucial step that leads to membrane weakening, placental rupture and early uterine contraction initiation. Maternal infections are believed to account for most of preterm delivery cases (25-40%), and uterine infections have been reported to be the leading cause of PTD. Vaginal microorganisms are capable of reaching the fetal membranes and inducing local proinflammatory response (chorioamnionitis) that ultimately results in PTD. Nonetheless, the treatment of symptomatic and/or asymptomatic uterine infections during pregnancy has revealed contradictory results in decreasing PTD rates. It has been speculated that other pathogens may come from different untreated focal infections in the body that reach the uterus through hematogenous dissemination and infect the maternal-fetal interface. In particular, *Campylobacter rectus* is a Gram negative anaerobe harbored in periodontitis-associated oral biofilms that has shown the competence to translocate to the fetoplacental unit and operate as a potential fetal infectious agent eliciting prematurity. Moreover, a number of clinical studies have found an association between periodontitis and preterm delivery.

Maternal periodontitis has been found to be associated with increased risk for fetal exposure to periodontal pathogens in PTD cases. Yet, the underlying biological mechanisms sustaining preterm delivery onset after *C. rectus* infection remain largely unknown. This dissertation hypothesized that *C rectus* induces a placental innate inflammatory response mediated by Toll-like receptors (TLRs). Our experimental data on animal models have demonstrated *C. rectus* ability to disseminate from distant sites of infection, to induce a local placental inflammatory response along with a fetal intrauterine growth restriction phenotype, and to upregulate TLR-4 expression in placental trophoblasts after infection. The experimental results here presented demonstrated the importance of TLRs in mediating proinflammatory phenotype both *in vitro* (human trophoblastic cell line) and *in vivo* (murine) infection models in response to *C. rectus* infection. Taken together, the results here presented will elucidate in part the maternal/fetal biological mechanisms leading to PTD in humans, bringing new insights, theories and health policies into the preterm delivery field.

Dedication

I dedicate this work to my parents Miguel and Luz Maria, in recognition for all their efforts to raise the person I am today, and for always encouraging me to aim high in my life.

I also dedicate this work to my wife Marisol, for her unconditional and endless love and support.

Acknowledgments

The Offenbacher laboratory has provided me with an excellent environment in which I could study the intricacies of the molecular aspects of periodontal pathogens affecting other body systems apart from the mouth. Dr. Offenbacher strikes the perfect balance between independent learning and strategic guidance. His trust in my abilities has given me the confidence to pursue a future career in dental academics and scientific research. I would like to thank "O" for training me as a scientist, teaching me to think critically, allowing me to participate in other projects and peer-reviewing activities, introducing me to his colleagues, helping me secure a postdoctoral training phase in clinical periodontology and taking an active interest in my scientific and personal development.

Another reason for the Offenbacher lab is a great place to do science is that it is populated by really nice people! My past and present co-workers have been some of my very good friends. Without exception the Offenbacher lab members have helped me troubleshoot potential problems, have pitched in to help me finish an experiment when needed, have provided kind acts of service to me and my wife, and have been great friends overall. Thanks for all the interesting conversations, for the fun times at RTP, for the invitations to potluck parties, and on and on.

I have an excellent committee composed of much respected researchers in the University. I thank Drs. Silvana Barros for all the hard work, guidance and dedication to

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Preface

Parts of this work were done in collaboration with other talented scientists. Chapter 2 represents a publication for which I was the first author. The paper was published previous to writing this thesis with the following citation:

> Arce RM, Barros SP, Wacker B, Peters B, Moss K, Offenbacher S. Increased TLR4 expression in murine placentas after oral infection with periodontal pathogens. *Placenta* 2009 Feb;30(2):156-62.

Permission to include the article in its entirety in a PhD dissertation was retained by the authors as explained at the authors' rights web page from Elsevier (publisher of Placenta).

Chapter 3 represents work done in collaboration with Dr. Patricia Diaz and Dr. Deborah Threadgill. I was the lead co-author on the paper with Dr. Diaz who generated the *in vivo* and *in vitro* invasion experiments in fulfillment of her Masters thesis in Periodontology. The paper was published previous to writing this thesis with the following citation:

> Arce RM, Diaz PI, Barros SP, Galloway P, Bobetsis Y, Threadgill D, Offenbacher S. 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.* 2010 Mar;84(2):145-53.

Permission to include the article in its entirety in a PhD dissertation was retained by the authors as explained at the authors' rights web page from Elsevier (publisher of JRI).

Chapter 4 represents unpublished research that was designed and performed primarily by myself under the guidance of Dr. Kathleen Caron and Dr. Steven Offenbacher.

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Abbreviations/Glossary

Abortion, Septic; Any type of abortion, induced or spontaneous, that is associated with infection of the uterus and its appendages. It is characterized by fever, uterine tenderness, and foul discharge.

Aggregatibacter actinomycetemcomitans: A species of gram-negative, facultative anaerobic spherical or rod-shaped bacteria that associates with species of actynomices in actinomycotic lesions and mainly found in severe infections in the oral cavity, mainly the periodontium.

Acute-Phase Proteins: Proteins that are secreted into the blood in increased or decreased quantities by hepatocytes in response to trauma, inflammation, or disease. These proteins can serve as inhibitors or mediators of the inflammatory processes. Certain acute-phase proteins have been used to diagnose and follow the course of diseases or as tumor markers.

Alveolar Bone Loss: Resorption or wasting of the tooth-supporting bone (alveolar process) in the maxilla or mandible.

Amniotic Fluid: A clear, yellowish liquid that envelopes the fetus inside the sac of amnion. In the first trimester, it is likely a transudate of maternal or fetal plasma. In the second trimester, amniotic fluid derives primarily from fetal lung and kidney. Cells or substances in this fluid can be removed for prenatal diagnostic tests (Amniocentesis).

Anaerobiosis: The complete absence, or (loosely) the paucity, of gaseous or dissolved elemental oxygen in a given place or environment.

Analysis of Variance (ANOVA): A statistical technique that isolates and assesses the contributions of categorical independent variables to variation in the mean of a continuous dependent variable.

Antagonists & inhibitors: Used with chemicals, drugs, and endogenous substances to indicate substances or agents which counteract their biological effects by any mechanism.

Antibodies: Immunoglobulin molecules having a specific amino acid sequence by virtue of which they interact only with the antigen (or a very similar shape) that induced their synthesis in cells of the lymphoid series (especially plasma cells).

Bacterial adhesiveness (adhesins): Cell-surface components or appendages of bacteria that facilitate adhesion to other cells or to inanimate surfaces. Most fimbriae of gram-negative bacteria function as adhesins.

Birth Weight: The mass or quantity of heaviness of an individual at BIRTH. It is expressed by units of pounds or kilograms.

Blastocyst: A post-morula pre-implantation mammalian embryo that develops from a 32-cell stage into a fluid-filled hollow ball of over a hundred cells. A blastocyst has two distinctive tissues. The outer layer of trophoblasts gives rise to extra-embryonic tissues. The inner cell mass gives rise to the embryonic disc and eventual embryo proper.

Campylobacter fetus (C. fetus): A species of bacteria present in man and many kinds of animals and birds, often causing infertility and/or abortion.

Campylobacter jejuni (C. jejuni): A species of bacteria that resemble small tightly coiled spirals. Its organisms are known to cause abortion in sheep and fever and enteritis in man and may be associated with enteric diseases of calves, lambs, and other animals.

Campylobacter rectus (C. rectus): A species of Campylobacter spp. isolated from cases of human periodontitis. It is a microaerophile, capable of respiring with OXYGEN.

Carboxymethylcellulose (CMC): A cellulose derivative which is a beta-(1,4)-D-glucopyranose polymer. It is used as a bulk laxative and as an emulsifier and thickener in cosmetics and pharmaceuticals and as a stabilizer for reagents.

CD14 (Cluster of differentiation 14): Glycolipid-anchored membrane glycoproteins expressed on cells of the myelomonocyte lineage including monocytes, macrophages, and some granulocytes. They function as receptors for the complex of lipopolysaccharide (LPS) and LPS-binding protein.

Cytokeratin 7: A class of fibrous proteins or scleroproteins that represents the principal constituent of epidermis. Type 7 is a type II keratin found associated with ductal epithelia, gastrointestinal epithelia and trophoblasts.

Chorioamnionitis: Inflammation of the placental membranes (chorion, amnion) and connected tissues such as fetal blood vessels and umbilical cord. It is often associated with intrauterine ascending infections during pregnancy.

Choriocarcinoma: A malignant metastatic form of trophoblastic tumors.

Chronic Periodontitis: Chronic inflammation and loss of periodontium that is associated with the amount of dental plaque or dental calculus present.

Cyclooxygenase 2: An inducibly-expressed subtype of prostaglandin-endoperoxide synthase. It plays an important role in many cellular processes and inflammation.

DNA, Complementary (cDNA): Single-stranded complementary DNA synthesized from an RNA template by the action of RNA-dependent DNA polymerase. cDNA (i.e.,

complementary DNA, not circular DNA, not C-DNA) is used in a variety of molecular cloning experiments as well as serving as a specific hybridization probe.

Endometrium: The mucous membrane lining of the uterine cavity that is hormonally responsive during the menstrual cycle and pregnancy.

Escherichia coli (E. coli): A species of gram-negative, facultative anaerobic, rod-shaped bacteria commonly found in the lower part of the intestine of warm-blooded animals.

Fetal Growth Restriction (FGR or IUGR): The failure of a fetus to attain its expected fetal growth at any gestational age.

Fetal Membranes (Premature Rupture, PROM, PPROM): Spontaneous tearing of the membranes surrounding the fetus any time before the onset of obstetric labor. Preterm PROM is membrane rupture before 37 weeks of gestation.

Fetal Weight: The weight of the fetus in utero. It is usually estimated by various formulas based on measurements made during prenatal ultrasonography.

Fluorescent Antibody Technique: Test for tissue antigen using either a direct method, by conjugation of antibody with fluorescent dye or an indirect method, by formation of antigen-antibody complex which is then labeled with fluorescein-conjugated anti-immunoglobulin antibody. The tissue is then examined by fluorescence microscopy.

Fusobacterium nucleatum (F. nucleatum): A species of gram-negative, anaerobic, rod-shaped bacteria isolated from the gingival margin and sulcus and from infections of the upper respiratory tract and pleural cavity.

Gene Expression: The phenotypic manifestation of a gene or genes by the processes of genetic transcription and genetic translation.

Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH): An NAD-dependent glyceraldehyde-3-phosphate dehydrogenase found in the cytosol of eukaryotes. It catalyzes the dehydrogenation and phosphorylation of Glyceraldehyde 3-phosphate to 3-phospho-D-glyceroyl phosphate, which is an important step in the glycolysis pathway. Its mRNA gene is commonly used as a housekeeping gene activity reference.

Gram-Negative Anaerobic Bacteria: A large group of anaerobic bacteria which show up as pink (negative) when treated by the Gram-staining method.

Granulocyte Colony-Stimulating Factor(GCSF): A glycoprotein of MW 25 kDa containing internal disulfide bonds. It induces the survival, proliferation, and differentiation of neutrophilic granulocyte precursor cells and functionally activates mature blood neutrophils. Among the family of colony-stimulating factors, G-CSF is the most potent inducer of terminal differentiation to granulocytes and macrophages of leukemic myeloid cell lines.

Hematoxylin and eosin (H&E stain): HE stain or hematoxylin and eosin stain is a staining method in histology.

Host-Pathogen Interactions: The interactions between a host and a pathogen, usually resulting in disease.

Immunoglobulin G: The major immunoglobulin isotype class in normal human serum. There are several isotype subclasses of IgG, for example, IgG1, IgG2A, and IgG2B.

Immunoglobulin M: A class of immunoglobulin bearing mu chains. IgM can fix complement. The name comes from its high molecular weight and originally being called a macroglobulin.

Interleukin-6 (IL-6): A cytokine that stimulates the growth and differentiation of B-lymphocytes and also mainly acts as a pro-inflammatory cytokine.

Kaplan-Meiers Estimate: A nonparametric method of compiling life tables or survival tables. It combines calculated probabilities of survival and estimates to allow for observations occurring beyond a measurement threshold, which are assumed to occur randomly. Time intervals are defined as ending each time an event occurs and are therefore unequal.

Labor, Premature: Onset of obstetric labor before term (term birth) but usually after the fetus has become viable. In humans, it occurs sometime during the 29th through 37th week of pregnancy.

Lipopolysaccharide (LPS): Lipid-containing polysaccharides which are endotoxins and important group-specific antigens. They are often derived from the cell wall of gram-negative bacteria and induce immunoglobulin secretion. The lipopolysaccharide molecule consists of three parts: LIPID A, core polysaccharide, and O-specific chains (O ANTIGENS).

Matrix Metalloproteinases (MMPs): Matrix metalloproteinases that are associated with the cell membrane, either through transmembrane domains or Glycosylphosphatidylinositol anchors. Membrane-type matrix metalloproteinases may act within the pericellular environment to influence the process of cell migration.

Mice, Inbred strains: Genetically identical individuals developed from brother and sister matings which have been carried out for twenty or more generations, or by parent x offspring matings carried out with certain restrictions. All animals within an inbred strain trace back to a common ancestor in the twentieth generation.

Mice, Knockout: Strains of mice in which certain genes of their genomes have been disrupted, or "knocked-out". To produce knockouts, using recombinant DNA technology, the normal DNA sequence of the gene being studied is altered to prevent synthesis of a normal gene product. Cloned cells in which this DNA alteration is successful are then injected into mouse embryos to produce chimeric mice. The chimeric mice are then bred to yield a strain in which all the cells of the mouse contain the disrupted gene.

Multiplicity of infection (MOI): ratio of infectious agents (e.g. phage or virus) to infection targets (e.g. cell). For example, when referring to a group of cells inoculated with infectious virus particles, the multiplicity of infection or MOI is the ratio defined by the number of infectious virus particles deposited in a well divided by the number of target cells present in that well.

Natural Killer Cells (NK cells): Bone marrow-derived lymphocytes that possess cytotoxic properties, classically directed against transformed and virus-infected cells. NK cells are not antigen specific. The cytotoxicity of natural killer cells is determined by the collective signaling of an array of inhibitory and stimulatory cell surface receptors.

NF-kappa B (NF-kB): Ubiquitous, inducible, nuclear transcriptional activator that binds to enhancer elements in many different cell types and is activated by pathogenic stimuli. The NF-kappa B complex is a heterodimer composed of two DNA-binding subunits: NF-kappa B1 and relA.

Pathogen-associated molecular patterns (PAMPs): are molecules associated with groups of pathogens, that are recognized by cells of the innate immune system. These molecules can be referred to as small molecular motifs conserved within a class of

microbes. They are recognized by Toll-like receptors (TLRs) and other pattern recognition receptors (PRRs) in both plants and animals.

Pattern recognition receptors (PRRs): A large family of cell surface receptors that bind conserved molecular structures present in pathogens. They play important roles in host defense by mediating cellular responses to pathogens.

Periodontal Diseases: Pathological processes involving the periodontium including the gum (gingiva), the alveolar bone (alveolar process), the dental cementum, and the periodontal ligament.

Placenta: A highly vascularized mammalian fetal-maternal organ and major site of transport of oxygen, nutrients, and fetal waste products. It includes a fetal portion (chorionic villi) derived from trophoblasts and a maternal portion (decidua) derived from the uterine endometrium.

Porphyromonas gingivalis (P. gingivalis): A species of gram-negative, anaerobic, rod-shaped bacteria originally classified within the bacteroides genus. This bacterium produces a cell-bound, oxygen-sensitive collagenase and is isolated from the human mouth.

Pre-Eclampsia: A complication of pregnancy, characterized by a complex of symptoms including maternal hypertension and proteinuria with or without pathological edema. Pre-eclampsia usually occurs after the 20th week of gestation, but may develop before this time in the presence of trophoblastic disease.

Pregnancy Complications: Conditions or pathological processes associated with pregnancy. They can occur during or after pregnancy, and range from minor discomforts

to serious diseases that require medical interventions. They include diseases in pregnant females, and pregnancies in females with diseases.

Pregnancy Outcome: Results of conception and ensuing pregnancy, including live birth; stillbirth; spontaneous abortion; induced abortion.

Preterm Delivery (PTD): childbirth before 37 weeks of pregnancy (259 days from the first day of the mother's last menstrual period, or 245 days after fertilization).

RNA, Messenger: RNA sequences that serve as templates for protein synthesis. Bacterial mRNAs are generally primary transcripts in that they do not require post-transcriptional processing. Eukaryotic mRNA is synthesized in the nucleus and must be exported to the cytoplasm for translation. Most eukaryotic mRNAs have a sequence of polyadenylic acid at the 3' end, referred to as the poly(A) tail. The function of this tail is not known for certain, but it may play a role in the export of mature mRNA from the nucleus as well as in helping stabilize some mRNA molecules by retarding their degradation in the cytoplasm.

Survival Analysis: A class of statistical procedures for estimating the survival function (function of time, starting with a population 100% well at a given time and providing the percentage of the population still well at later times). The survival analysis is then used for making inferences about the effects of treatments, prognostic factors, exposures, and other covariates on the function.

Toll-Like Receptors (TLRs): A family of pattern recognition receptors characterized by an extracellular leucine-rich domain and a cytoplasmic domain that share homology with the Interleukin 1 receptor and the Drosophila toll protein. Following pathogen

recognition, toll-like receptors recruit and activate a variety of signal transducing adaptor proteins.

Toll-Like Receptor 4 (TLR4): A pattern recognition receptor that interacts with lymphocyte antigen 96 and lipopolysaccharides. It mediates cellular responses to GRAM negative bacteria.

Trophoblasts: Cells lining the outside of the blastocysts. After binding to the endometrium, trophoblasts develop into two distinct layers, an inner layer of mononuclear cytotrophoblasts and an outer layer of continuous multinuclear cytoplasm, the syncytiotrophoblasts, which form the early fetal-maternal interface (placental).

Tumor Necrosis Factor-alpha (TNF-α): Serum glycoprotein produced by activated macrophages and other mammalian mononuclear leukocytes. It has necrotizing activity against tumor cell lines and increases ability to reject tumor transplants. Also known as TNF-alpha, it is only 30% homologous to TNF-beta (Lymphotoxin), but they share TNF receptors.

World Health Organization: A specialized agency of the United Nations designed as a coordinating authority on international health work; its aim is to promote the attainment of the highest possible level of health by all peoples.

Kruskal-Wallis H Statistic: A class of statistical methods applicable to a large set of probability distributions used to test for correlation, location, independence, etc. In most nonparametric statistical tests, the original scores or observations are replaced by another variable containing less information.

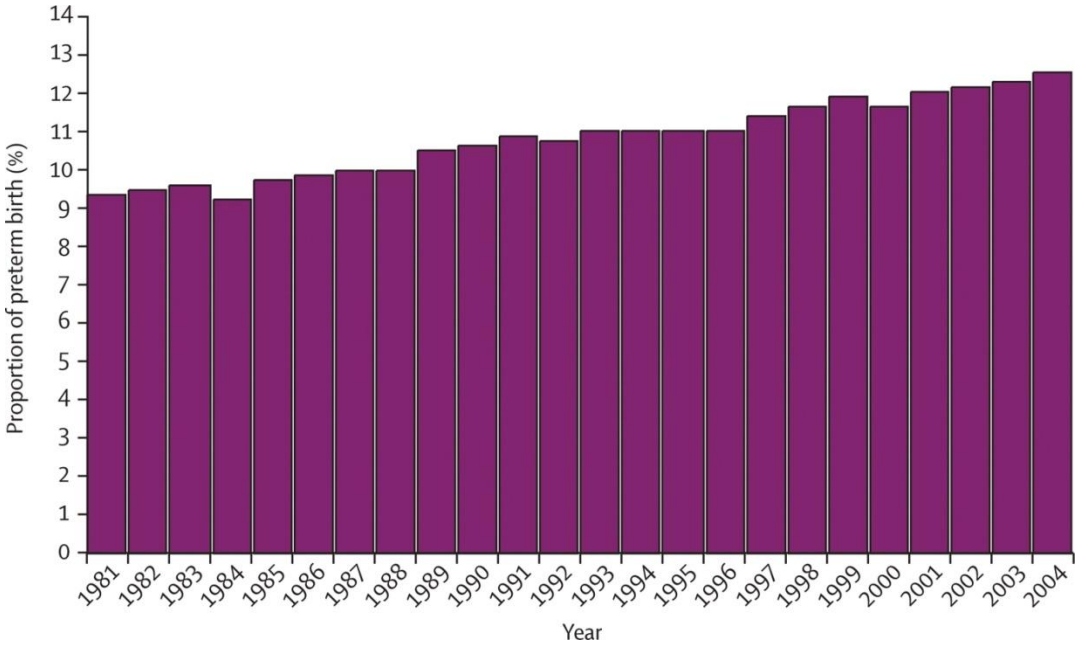
1. Chapter 1. Introduction

Preterm delivery (PTD): Epidemiology and definitions

Preterm delivery is the major cause of neonatal mortality and morbidity and is associated with short-term (low birth weight) and long-term adverse sequelae (cerebral palsy, hearing and learning problems)(MacDorman et al. 2005). The World Health Organization defines PTD as birth at less than 37 completed gestational weeks(1970); however, an additional definition of very preterm delivery (birth at <32 weeks) has been proposed based on higher morbidity/mortality associated rates(Kramer et al. 2000). The prevalence of PTD around the world ranges from 5-9% in Europe, 12-13% in the USA (Figure 1.1) and up to 34% in India(Goldenberg et al. 2008, Ananth & Vintzileos 2006). Broadly speaking, preterm deliveries are believed to account for up to 75-85% of the early neonatal deaths not due to lethal congenital deformities around the world(2004, McCormick 1985). In the United States, extremely preterm infants have been reported to account for 49%-58% of neonatal deaths during 1989-2001 accompanied by significant racial/ethnic associated factors such as lack of prenatal care or social support, stress and bacterial vaginosis. Notably, USA neonatal death rates have declined over the last decades, probably due to medical advances such as surfactant therapy (to improve infant lung maturity), folic acid consumption (to reduce neural tube defects) and antimicrobial therapy (to control infectious episodes during pregnancy)(Simcox et al. 2007). In fact, Preventive therapies with antibiotics have emerged as a prophylactic strategy to decrease PDT rates in industrialized countries; however there is conflicting evidence coming from randomized clinical trials evaluating the real benefits of antibiotic intake by pregnant

women(Laudanski et al. 2007). Researchers have proposed that the contradictory results could be related to the fact that some human subclinical infections do not respond well to wide-spectrum antimicrobial therapy, raising the possibility that common non-treated infections (including periodontal disease) may play a role in PTD pathogenesis(Vidaeff & Ramin 2006, Offenbacher et al. 1996).

Figure 1.1 Percentage of all births classified as preterm in the USA, 1981-2004.



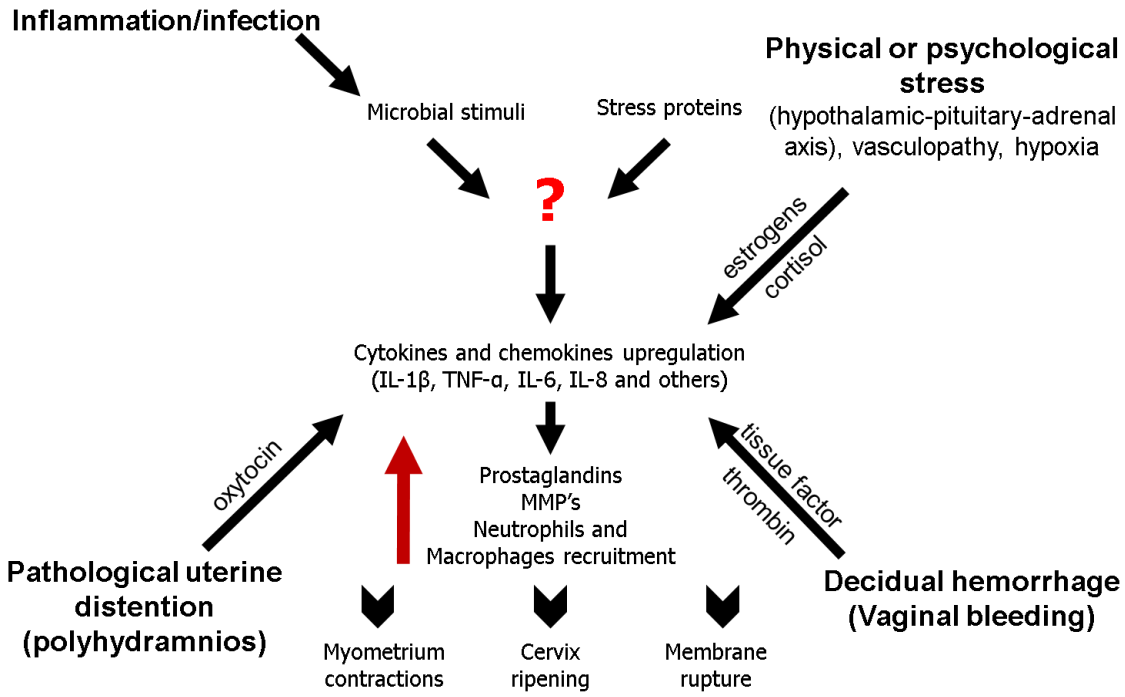
Despite recent advances in the understanding of preterm delivery, there is an increase in the proportions of preterm birth (%) in the last 20 years. Adapted from (Goldenberg et al. 2008). Source: Martin JA, Kochanek KD, Strobino DM, Guyer B, MacDorman MF. Annual summary of vital statistics—2003. Pediatrics 2005; 115: 619–34.

PTD pathogenesis: infection, inflammation and the role of placental Toll like receptors

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). Even though the precise triggering mechanism has not been established, the development of a proinflammatory condition is a common pathway that centralizes all multiple risk factors(Romero et al. 1994) (Figure 1.2). Histologically, local inflammatory features at the fetoplacental unit are termed as chorioamnionitis, (initially confined at the choriodecidual level) which activate the decidua and the fetal membranes to produce a number of cytokines, including Tumor Necrosis factor alpha (TNF-α), Interleukin-1 beta (IL-1β), Interleukin-6 (IL-6), Interleukin-8 (IL-8), and Granulocyte Colony-stimulating factor (GCSF)(Gomez et al. 1997). These cytokines stimulate prostaglandins synthesis/release and also initiate neutrophil chemotaxis, infiltration, and activation. The inflammatory process continues with the synthesis and release of metalloproteinases (MMP's) and other bioactive substances; the prostaglandins stimulate uterine contractions while the MMP's degrades the chorioamniotic membranes, leading to either spontaneous preterm labor with intact membranes or preterm premature rupture of the membranes-PPROM(Goldenberg et al. 2000).

Particularly, uterine infections account for 25–40% of preterm births and they are strongly linked with the inflammatory pathway onset. Uterine infections are known to upregulate the production of local pro-inflammatory cytokines,

Figure 1.2 The pathogenesis of human preterm delivery.



Model proposed by (Gomez et al. 1997). The authors proposed that the initiation of human parturition in the presence of infection is controlled by the host. Systemic maternal infections such as pyelonephritis or localized infections such as deciduitis can trigger parturition by the activation of the monocyte and macrophage system in peripheral blood and human decidua. Preterm labor can be the result of events that occur when the intrauterine or maternal environment is hostile and threaten the survival of the fetal-maternal pair. Preterm labor and preterm premature rupture of membranes (PPROM) can be considered expressions of the same basic phenomenon: activation of the host-defense macrophage system. Adapted from Gomez R, Romero R, Edwin SS, David C. Pathogenesis of preterm labor and preterm premature rupture of membranes associated with intraamniotic infection. *Infect Dis Clin North Am* 1997 11(1):135-76.

metalloproteinases and prostaglandins that lead to membrane weakening, early membrane rupture and uterine contraction initiation(Shoji et al. 2007). Uterine infections usually take advantage of ascending mechanisms, which can be described briefly in three different stages: the first stage consists of an overgrowth of anaerobic microorganisms or the presence of pathologic organisms (i.e., *Neisseria gonorrhoeae* or *Ureaplasma urealyticum*) in the vagina and cervix. Bacterial vaginosis may be an early manifestation of stage 1. Once microorganisms ascend and gain access to the intrauterine cavity, they may reside and colonize the decidua (stage 2). A localized inflammatory reaction leads to deciduitis and spreads to chorionitis. The infection may invade the fetal vessels (choriovasculitis) or proceed through the amnion (amnionitis) into the amniotic cavity and lead to an intraamniotic infection (stage 3). The most advanced and serious stage of ascending intrauterine infection is fetal infection (stage 4)(Gomez et al. 1997).

If placental tissues ultimately present an inflammatory phenotype in response to multiple PTD-associated triggering factors, then proinflammatory responses are likely to be initiated by the host innate immune system. Toll-like receptors (TLRs) are a family of transmembrane proteins that have a primary role in pathogen recognition and innate immunity initiation(Brikos & O'Neill 2008). TLRs receptors bind to several microbial components or end-products known as pathogen-associated molecular patterns (PAMPs), which include peptidoglycans, lipoteichoic acid, flagellin, double-stranded viral RNA, unmethylated bacterial DNA and lipopolysaccharide (LPS) among others (Table 1.1). After binding and recognition,

Table 1-1 Summary of known mammalian TLRs.

Receptor	Ligand(s)	Ligand location	Adapter(s)	Location	Cell types
TLR 1	multiple triacyl lipopeptides	Bacteria	MyD88/MAL	cell surface	monocytes/macrophages
					a subset of dendritic cells
					B lymphocytes
TLR 2	multiple glycolipids	Bacteria	MyD88/MAL	cell surface	monocytes/macrophages
	multiple lipopeptides	Bacteria			Myeloid dendritic cells[19]
	multiple lipoproteins	Bacteria			Mast cells
	lipoteichoic acid	Bacteria			
	HSP70	Host cells			
	zymosan (Beta-glucan)	Fungi			
	Numerous others				
TLR 3	double-stranded RNA, poly I:C	viruses	TRIF	cell compartment	Dendritic cells
					B lymphocytes
TLR 4	lipopolysaccharide	Gram-negative bacteria	MyD88/MAL/TRIF/TRAM	cell surface	monocytes/macrophages
	several heat shock proteins	Bacteria and host cells			Myeloid dendritic cells[19]
	fibrinogen	host cells			Mast cells
	heparan sulfate fragments	host cells			Intestinal epithelium
	hyaluronic acid fragments	host cells			
	nickel				
	Numerous others				
TLR 5	flagellin	Bacteria	MyD88	cell surface	monocyte/macrophages
					a subset of dendritic cells
					Intestinal epithelium
TLR 6	multiple diacyl lipopeptides	Mycoplasma	MyD88/MAL	cell surface	monocytes/macrophages
					Mast cells
					B lymphocytes
TLR 7	imidazoquinoline	small synthetic compounds	MyD88	cell compartment	monocytes/macrophages
	loxoribine (a guanosine analogue)				Plasmacytoid dendritic cells
	bropirimine				B lymphocytes
	single-stranded RNA				
TLR 8	small synthetic compounds; single-stranded RNA		MyD88	cell compartment	monocytes/macrophages
					a subset of dendritic cells
					Mast cells
TLR 9	unmethylated CpG Oligodeoxynucleotide DNA	Bacteria	MyD88	cell compartment	monocytes/macrophages
					Plasmacytoid dendritic cells
					B lymphocytes
TLR 10	unknown	unknown	unknown	cell surface	monocytes/macrophages
					B lymphocytes
TLR 11	Profilin	Toxoplasma gondii	MyD88	cell compartment	monocytes/macrophages
					liver cells
					kidney
					urinary bladder epithelium

Adapted from Waltenbaugh C, Doan T, Melvold R, Viselli S (2008). Immunology. Lippincott's Illustrated reviews. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins. pp. 17.

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 & 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). TLRs are highly involved in responding to inflammatory processes in the presence or absence of infection. In particular, TLRs are thought to be a critical component of the innate immune response during pregnancy, which have significant implications for the success or failure of pregnancies in both early and late gestation(Patni et al. 2007). To date, 13 mammalian Toll-like receptors homologues have been identified and designated (Table 1.1), and their expression has been described in the human placenta, mostly at the dominant cell type: the trophoblast(Abrahams et al. 2004, Holmlund et al. 2002, Kumazaki et al. 2004). Trophoblasts are the first cells to differentiate from the outer layer of the blastocyst, which are believed to be in charge of endometrial implantation and placental development(Cohen & Bischof 2007). Nonetheless, trophoblasts have also been proposed to be involved in coordinating the immune response during both processes(Mor 2008). For example, trophoblast are believed to regulate immune cells migration (macrophages and NK cells) to the endometrial implantation site by means of TLRs activation and subsequent chemokines production(Abrahams et al. 2005); abnormalities in decidual TLRs expression or function have been linked to abnormal placentation, inflammation, and adverse pregnancy outcomes(Krikun et al. 2007).

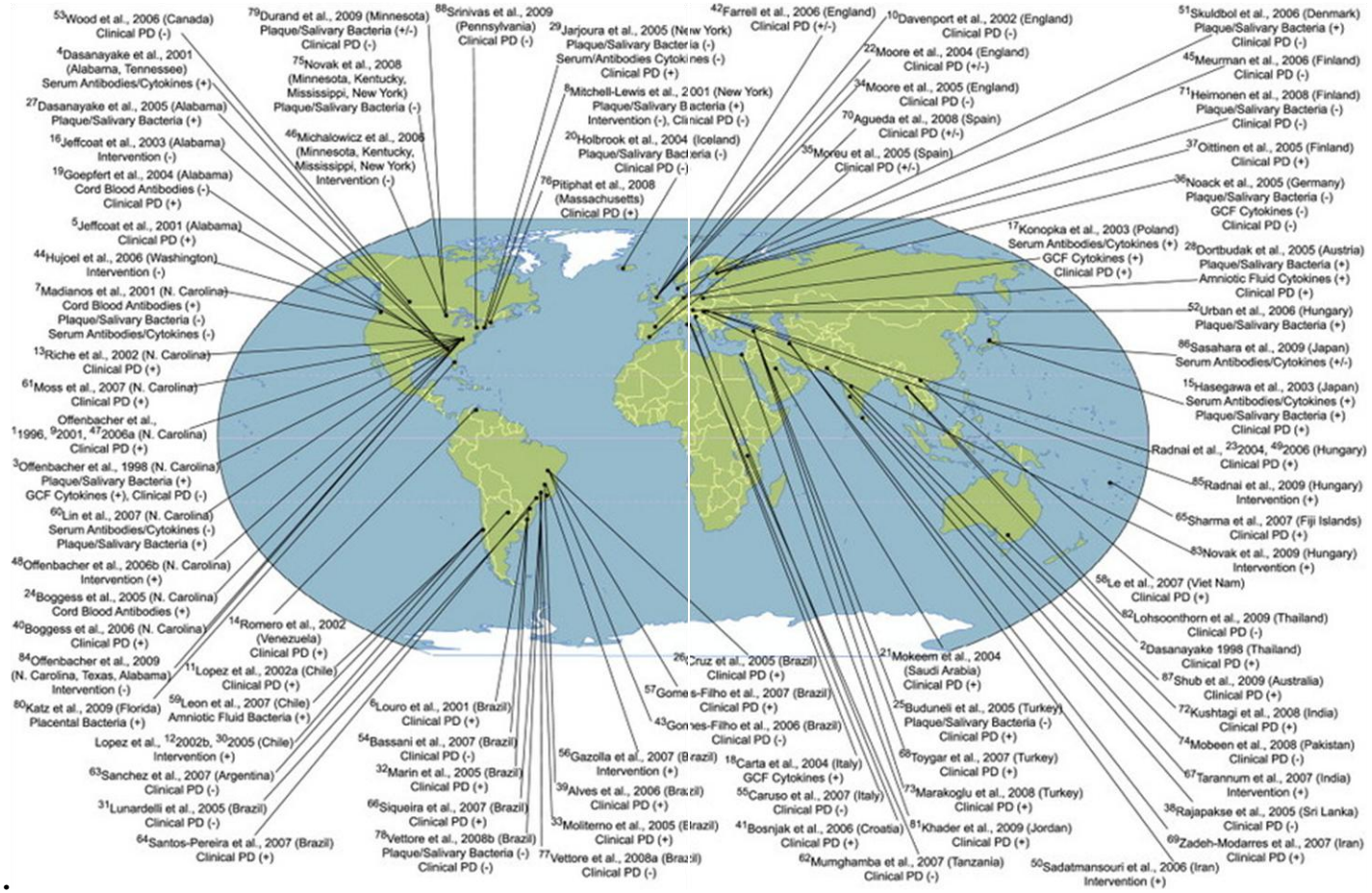
While TLRs are nowadays recognized to play an important role in the regulation of immune responses during pregnancy, the factors that regulate and affect TLR expression and function are poorly understood. Even though TLRs widely participate in placental immunological surveillance, the fact that specific toll like receptors or associated pathways prevail during placental inflammation may give new insights in understanding PTD pathogenesis. The selective activation of specific TLRs and TLR-associated pathways in response to local infection to several pathogens, including bacteria of oral origin, may bring new ideas in therapeutic interventions in the near future.

The Association of Periodontal disease and preterm delivery

Periodontal disease (gingivitis and periodontitis) is one of the most common chronic infections affecting up to 50% of humans(Albandar 2002). Periodontitis is initiated when specific microorganisms accumulate between the teeth and gums, forming bacterial biofilms commonly known as dental plaque. The body reacts to dental biofilms by activating a local inflammatory response that -at some susceptible patients- is unsuccessful in controlling or eliminating the infection. With time, the inflammatory response remains chronic and ultimately leads to periodontal connective tissue resorption (alveolar bone loss) and tooth loss(Madianos et al. 2005).

Periodontal disease is not closely associated with pain experience, and patients usually carry the disease for several years until noticing end-stage clinical signs (discomfort, breath malodor, gingival bleeding and tooth mobility)(Gilbert & Nuttall 1999, Pitiphat et al. 2002, Cunha-Cruz et al. 2007). Hence, the characteristic "silent" and chronic nature of periodontitis has led investigators to hypothesize that the disease course may serve as a chronic focal infection source for different systemic diseases(Li et al. 2000). In particular, periodontal diseases have been found to be an independent risk factor for pregnancy-related complications like preterm delivery, low birth weight and preeclampsia, after adjusting for other known obstetric risk factors(Riche et al. 2002, Offenbacher et al. 2006, Lin et al. 2007, Contreras et al. 2006, Scannapieco et al. 2010) (Figure 1.3). During the prolonged oral inflammatory response, periodontopathogenic bacteria and related virulence factors invade

Figure 1.3 Published studies on the association between periodontal disease and pregnancy outcomes



Adapted from (Scannapieco et al. 2010)**.**

periodontal tissues, enter the blood stream by means of transient bacteremias(Lafaurie et al. 2007) and disseminate throughout systemic organs. In fact, important periodontal pathogens including *Porphyromonas gingivalis* have been detected in atherosclerotic plaques(Chiu 1999, Ford et al. 2006, Haraszthy et al. 2000, Fiehn et al. 2005, Kozarov et al. 2006) and in the amniotic fluid of pregnant women with a diagnosis of threatened premature labor(Leon et al. 2007).

Campylobacter species and their role in uterine and oral infections

Translocation of oral bacteria to the developing fetus has also been demonstrated in umbilical cord blood samples from preterm deliveries by detecting maternal immunoglobulin G (IgG) as well as fetal immunoglobulin M (IgM) to one or more specific oral pathogens. In particular, *Campylobacter rectus* has been reported to be significantly prevalent in preterm deliveries based on low maternal IgG and high fetal IgM titers(Madianos et al. 2001), suggesting that *C. rectus* could act as a fetal infectious agent eliciting prematurity. *C. rectus* is an orally-exclusive Gram negative anaerobe and motile bacterium that plays a pathogenic role in human periodontitis(Rams et al. 1993, Yokoyama et al. 2008). *Campylobacter* species show a wide array of virulence factors including surface layer (S-layer) proteins(Miyamoto et al. 1998, Thompson 2002) (involved in phagocytic and bactericidal resistance); cytolethal distending toxin (CDT)(Gillespie et al. 1993) and RTX proteins(Braun et al. 1999) (pore-forming protein toxins similar to hemolysins and leucotoxins); GroEL-like proteins(Hinode et al. 1998) (heat-sock protein activity), flagella (motility) and lipopolysaccharide (LPS)(Ogura et al. 1995, Ogura et al. 1996, Takiguchi et al. 1996). Interestingly, *C. rectus* is part of the Campylobacteraceae family which has been associated with other diseases showing important mechanistic similarities: for example, *Campylobacter jejuni* is the most common causal agent of acute gastroenteritis (diarrhea) in the USA(Allos 2001) and *Campylobacter fetus* is a significant causal agent of sheep and cattle abortion due to a marked tropism for placental tissues(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 intrauterine growth restriction(O'Sullivan et al. 1988a, O'Sullivan et al. 1988b). Noteworthy, our *in vivo C. rectus* infection experiments in pregnant mice have consistently shown maternal inflammatory serum markers upregulation, dissemination to placental tissues and consequent fetal intrauterine growth restriction(Offenbacher et al. 2005, Yeo et al. 2005) and placental structural alterations as well as important gene expression changes(Bobetsis et al. 2007).

In summary, the evidence here discussed suggest that periodontal disease may facilitate the oral-fetal hematogenous translocation of oral pathogens probably due to increased bacterial load in the oral cavity, the altered immune response in the susceptible host and the chronic inflammatory disease course. In addition, the oral pathogen *Campylobacter rectus* shows important virulence factors that may elicit prematurity in humans. Therefore, we believe that periodontal disease and related pathogens have unique features that are linked to pregnancy complications and the research here presented will lead to new knowledge, insights, theories and health policies in the preterm delivery field in the upcoming future.

2. Chapter 2. Increased TLR4 expression in murine placentas after oral infection with periodontal pathogens.

Abstract

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

abnormal regulation of placental TLR4 may give new insights into how maternal periodontitis and periodontal pathogens might be linked to placental inflammation and preterm birth pathogenesis.

Introduction

Preterm birth (birth at ≤37 completed gestational weeks) is the major cause of neonatal mortality/morbidity in the world, accounting for up to 75-85% of the early neonatal deaths as well as to high rates of short-term (low birth weight) and long-term adverse sequelae (hearing/learning problems and cerebral palsy) (McCormick 1985). The pathogenesis of preterm birth is thought to be multi-factorial, possibly initiated by multiple mechanisms including infection, uteroplacental ischemia, hemorrhage, stress and other immunologically mediated processes (Romero et al. 2006); however, the development of a pro-inflammatory condition is a common effector pathway that centralizes all multiple risk factors (Romero et al. 1994). In particular, uterine infections may account for 25–40% of preterm births and they are strongly linked with a pro-inflammatory systemic state. For example, uterine infections are known to upregulate the production of local pro-inflammatory cytokines, metalloproteinases and prostaglandins that lead to membrane weakening, early membrane rupture and uterine contraction initiation (Shoji et al. 2007). Uterine infections usually take advantage of ascending mechanisms, which are originated from vaginal infections (i.e. *Neisseria gonorrhoeae* or *Ureaplasma urealyticum*) that lead to intrauterine cavity access, decidua colonization, localized inflammation onset (or chorioamnionitis), intraamniotic infection and ultimately fetal infection (Gomez et al. 1997).

Nonetheless, other sources of infection including the oral cavity have been proposed to facilitate the hematogenous transmission of pathogens that affect normal

pregnancy development (Hill 1998). In particular, periodontal diseases (gingivitis and periodontitis) are part of the most common chronic infections affecting up to 50% of humans (Albandar 2002) and has been found to be an independent putative risk factor for pregnancy-related complications such as preterm births, low birth weight and preeclampsia, after adjusting for other known obstetric risk factors (Offenbacher et al. 2006, Contreras et al. 2006). Periodontitis is initiated when specific microorganisms accumulate between the teeth and gums, forming bacterial biofilms commonly known as dental plaque. The body reacts to dental biofilms by activating the oral mucosal inflammatory response that –in some susceptible patients- is unsuccessful in controlling the infection. With time, the inflammatory response remains chronic and ultimately leads to periodontal connective tissue resorption (alveolar bone loss) and tooth loss (Madianos et al. 2005). During prolonged periodontal inflammation, periodontal pathogens and related virulence factors invade periodontal tissues, enter the blood stream by means of transient bacteremias (Forner et al. 2006) and disseminate throughout different systemic organs. In fact, important periodontal pathogens have been detected in human placentas of women with preeclampsia (Barak et al. 2007) and in the amniotic fluid of pregnant women with a diagnosis of premature labor (Leon et al. 2007) or premature labor with intact membranes (Chaim & Mazor 1992, Gardella et al. 2004).

Fetal exposure to periodontal pathogens from maternal oral biofilms has also been demonstrated in umbilical cord blood samples from preterm births by detecting maternal immunoglobulin G (IgG) as well as fetal immunoglobulin M (IgM) to one

or more specific oral pathogens. In particular, mothers with a low IgG response to *P. gingivalis* combined with a high fetal IgM response to *C. rectus* showed the highest rate of preterm deliveries (66.7%) among 812 deliveries from a cohort study of pregnant mothers (adjusted OR 10.3; P<0.0001) (Madianos et al. 2001), suggesting that *P. gingivalis* and *C. rectus* could act as fetal infectious agents eliciting complications during pregnancy. *C. rectus* is an Gram negative anaerobe and motile bacterium unique to the oral cavity that is phylogenetically related to *H. pylori* and is associated with ulceration of the periodontal attachment apparatus (Rams et al. 1993). Interestingly, other *Campylobacter* species are known to be a significant causal agent of sheep and cattle abortion due to a marked tropism for placental tissues (Fujihara et al. 2006). In animal models, *C jejuni* and *C. fetus* infections result in impaired development and fetal growth restriction (FGR) (O'Sullivan et al. 1988b).We have previously reported that a subcutaneous infection *with C. rectus* in pregnant mice disseminates to placental tissues and induces FGR (Offenbacher et al. 2005, Yeo et al. 2005), placental inflammation and structural alterations. (Bobetsis et al. 2007). Likewise, animal experiments using *Porphyromonas gingivalis* in a subcutaneous infection model have shown increased maternal inflammatory serum markers (Interleukin-6 and tumor necrosis factor alpha TNF-α), and increased fetal biochemical markers of placental inflammation (prostaglandin E_2) in murine amniotic fluid (Lin et al. 2003b, Lin et al. 2003a).

Placental infection and subsequent inflammation have been associated with preterm labor, so the biological pathways related to early inflammatory responses are

likely to mediate pathogenesis. *Toll-like* receptors (TLRs) are pattern recognition receptors that play a key role in the innate inflammatory response (Uematsu & Akira 2006) and have been proposed to play important roles in pregnancy maintenance, placental immune protection and delivery initiation (Patni et al. 2007). To date, a total of 10 human and 12 murine TLRs have been described. In general, TLRs can be categorized into two main groups based on their ligands: the first group consists of TLR1, 2, 4, and 6 which recognize bacterial molecules such as lipopolysaccharide (LPS), lipoteichoic acid and peptidoglycan. The second group consists of TLR3, 7, 8, and 9 that recognize pathogen-associated nucleic acids patterns (Brikos & O'Neill 2008). Here in we focused on TLR4 which is selectively activated by Gram negative LPS, in conjunction with CD14 (Pandey & Agrawal 2006). Since *P. gingivalis* and *C. rectus* are Gram negative periodontal pathogens, the main objective of this study was to determine whether an oral infection with *C. rectus* and *C. rectus*/*P. gingivalis* combined infection could affect fetal growth, fecundity and induce placental inflammation along with enhanced expression of TLR4 in a timed-pregnancy murine model.

Materials and Methods

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

Bacterial strains and inoculum preparation: *C. rectus* 314 and *P. gingivalis* A7436 aliquots were maintained in Wilkins Chalgren anaerobic broth medium (WC broth; DSMZ, Braunschweig, Germany) containing 10% skim milk at -80°C. *C. rectus* aliquots were reconstituted on PRAS ETSA plates (Enriched Tryptic Soy Agar from Anaerobe Systems, Morgan Hill CA) and *P. gingivalis* aliquots on Anaerobic Reducible Blood Agar (from Remel, Lenexa KA). For experiments, bacteria were anaerobically grown under 5% 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 concentrations were determined by spectrophotometry (Cecil Instruments, Cambridge, UK) with a measured optical density at 600 nm (*C. rectus*) and 660 nm (*P. gingivalis*) corresponding to $1x10^9$ bacteria/ml respectively. Finally, all oral preparations were adjusted accordingly to keep the same concentration during oral infection experiments.

Oral infection protocol: The oral infection model of experimental periodontitis has been described elsewhere (Lalla et al. 2003). This model involves a pretreatment phase with antibiotics to suppress the oral flora to permit the colonization of exogenously applied human oral bacterial strains and the use of carboxymethylcellulose (CMC) as a carrier to facilitate bacterial colonization. Briefly, before experiment baseline 40 female Balb/C mice were pretreated for 4 days with Kanamycin/Ampicillin (50mg/kg-25mg/kg) followed by a 3-day antibiotic wash out period, and then randomly assigned to experimental groups (Table 2.1 and Figure 2.1). Mice were topically infected in the oral cavity with $1x10^{10}$ live bacterial units/100uL in a phosphate-buffered saline (PBS) and 2% CMC preparation on a daily basis. Controls included a blank group (same diet without oral infection) and a negative control group (CMC application without bacteria).

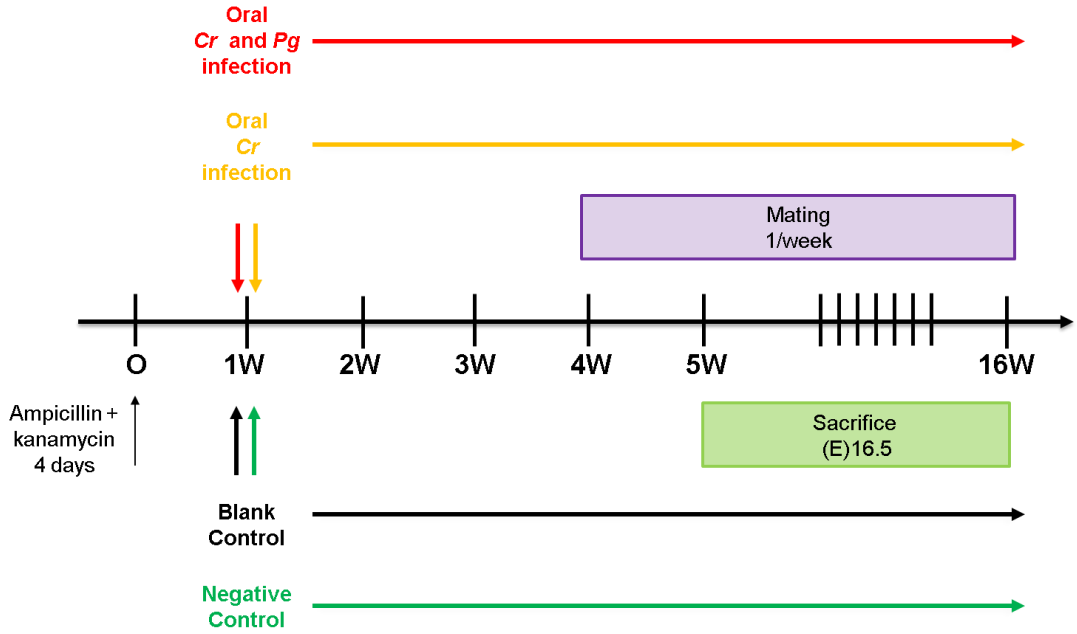
Placental histology and Immunofluorescence: Placentas were fixed in 4% paraformaldehyde, bisected sagittally, processed and embedded in paraffin. Sections (~5 μm) were stained using hematoxylin/eosin (H&E) for structural analysis; other sections were processed for immunofluorescence. Briefly, tissue antigens were rescued with Safeclear® (Fisher Protocol, Fair Lawn NJ) for 20 minutes and washed

Table 2-1 Experimental groups.

Experimental Group	N	Description
Blank control	8	High-glucose soft chow
Negative control	8	High-glucose soft chow CMC topical application
Campylobacter rectus infection	12	High-glucose soft chow CMC + *C. rectus* ($1x10^{10}$ CFU)
Campylobacter rectus and *Porphyromonas gingivalis* infection	12	High-glucose soft chow CMC + *C. rectus* ($1x10^{10}$ CFU) CMC + *P. gingivalis* ($1x10^{10}$ CFU)

All groups were given ad libitum soft chow enriched with a dextrose solution (30%) as a plaque-promoting diet during 16 weeks of experimentation. CMC = 2% carboxymethylcellulose in PBS; CFU = colony forming units.

Figure 2.1 Experiment timeline.



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

under serial ethanol concentrations. After washing in 0.2% Triton/PBS, sections were incubated in 10% non-immune goat serum and bovine serum albumin in PBS for 1 hour. Rabbit anti-TLR4 monoclonal antibody (Zymed, Invitrogen, Carlsbad CA) and mouse anti-mouse Cytokeratin 7 monoclonal antibody (RCK105 from Abcam, Cambridge, UK) were incubated overnight on a 1:50 concentration. Cytokeratin 7 was chosen as a trophoblast marker following the recommendations of the workshop report on cell culture models of trophoblasts (Frank et al. 2000). After vigorous washing, secondary biotinylated antibodies were applied for 1 hour (Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti–mouse IgG from Molecular Probes, Invitrogen, Carlsbad CA) for 1h. Sections were washed in 0.2% Triton/PBS, mounted and coverslipped with Vectashield (Vector labs, Burlingame CA). All stained sections were analyzed and photographed under confocal microscopy (LSM5, Carl Zeiss, Thornwood NY).

Quantitative RT-PCR for TLR4: Total RNA was isolated from all placental tissues (n=135) with the use of the RNeasy Mini Kit (Qiagen). cDNA from 2 µg of total RNA was synthesized using the Omniscript Kit (Qiagen) and random decamer primers. Real-time PCR was performed with 1 µL cDNA, TaqMan Universal PCR mix, and 20X primer (Mm00445273_m1 from Applied Biosystems, Foster City, CA), in a 7000 Sequence Detection System (ABI Prism, Applied Biosystems). Reactions were performed in duplicates and in two independent times. The Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) gene was used as an endogenous control (housekeeping gene). Results were evaluated using the delta-delta Ct method, where

delta Ct was calculated as (TLR4 Ct) − (*GAPDH* Ct), and the relative quantity of TLR4 mRNA expression was calculated by the delta–delta Ct as $2^{-[(\text{infected sample delta Ct})-(\text{control sample delta Ct})]}$.

Statistical analysis: A sample size of 8 mice per group was calculated [power (1-β) of >0.90% with alpha-error threshold of (α)=0.05)] based on our previous results on fetal growth restriction after *C. rectus* systemic infection (Yeo et al. 2005). Categorical variables (number of pregnancies and number of fetuses/resorptions per group) were summarized using frequencies and percentages, and continuous variables (fetal weight/length and mRNA levels) were described using means and standard errors. Distributions of fetuses/resorptions were compared using the Chi-square test. Mean fetal weight and length values as well as mRNA levels were compared using Analysis of Variance (*ANOVA*). To determine whether oral infection correlated with FGR and resorption induction, a general linear regression model (GLM procedure) was used to control for clustering within litters per group. Also, resorptions were considered as zero (value=0) to account for their impact on the average litter weight and length. Kaplan-Meier estimation analysis was used to evaluate cumulative pregnancy events at 16 weeks. Cox proportional hazards ratios were used to determine the risk of no pregnancies over time after infection. If no significant differences were observed between the control groups (blank and negative), then controls were regrouped as non-infected group for analysis. The threshold for statistical significance was set at a P-value less than 0.05. All analyses were performed using SAS v.9.2. (SAS Institute, Cary, NC).

Results

Oral infection with *C. rectus* and *P. gingivalis* induced growth restricted fetuses and more fetal resorptions. The murine pregnancy events and fetal outcomes after oral infection with periodontal pathogens are depicted in Table 2.2. The number of mice which became pregnant during the 16 week infection/mating protocol were significantly different among all groups ($P<0.05$, Chi-square test) as well as in the distribution of fetuses and resorptions ($P<0.01$). On average, fetuses from infected mice were smaller and lighter, although fetal lengths were not statistically different among all groups ($P=0.08$, *ANOVA*). When control groups were compared, no significant differences were observed for number of fetuses/resorptions [27(96.4%) vs. 41(80.4%), $P=0.27$, Chi-square], and for fetal weight values [0.47±0.012 vs. 0.39±0.035, $P=0.87$, *ANOVA*, blank and negative groups respectively]. However, when fetal weight values from infected mice were compared to those from non-infected mice (regrouped controls), a statistically significant decrease was observed for the *C. rectus* and *P. gingivalis* infected group ($P<0.05$, *ANOVA*, Figure 2). Overall, the general linear regression model which adjusted for clustering within litters was non-significantly associated with FGR induction ($P=0.28$, GLM procedure).

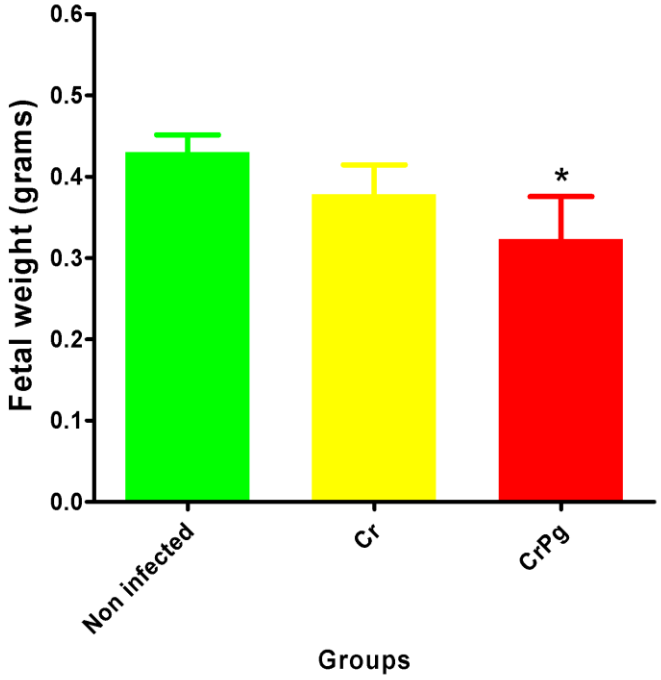
Murine fecundity was affected after oral infection. Murine fecundity was significantly different among experimental groups (Table 2.3 and Figure 2.2). 68.8% of non-infected mice got pregnant; however fecundity decreased to 58.3% in the *C. rectus* infected group and only 16.7% of mice receiving *C. rectus* and *P. gingivalis*

Table 2-2 Pregnancy events and fetal outcomes after oral infection.

Experimental Group	N	Pregnancy events [n (%)]	Fetuses [n (%)]	Resorptions [n (%)]	Fetal length (mm) (mean±SE)	Fetal weight (grams) (mean±SE)
Blank	8	5(62.5)	27(96.4)	1(3.6)	1.41±0.02	0.47±0.012
Negative	8	6(75.0)	41(80.4)	10(19.6)	1.18±0.103	0.39±0.035
C. rectus	12	7(58.3)	55(73.3)	20(26.7)	1.09±0.104	0.37±0.036
C. rectus and P. gingivalis	12	2(16.7)	12(70.6)	5(29.4)	1.02±0.265	0.32±0.052

Distribution of pregnancies, number of fetuses and resorptions were significantly different among groups ($P < 0.01$, Chi-square). Viable fetuses from infected mice tended to be smaller and lighter on average, but fetal lengths were not different among all groups ($P = 0.08$, ANOVA).

Figure 2.2 Oral infection induces low weight fetuses.



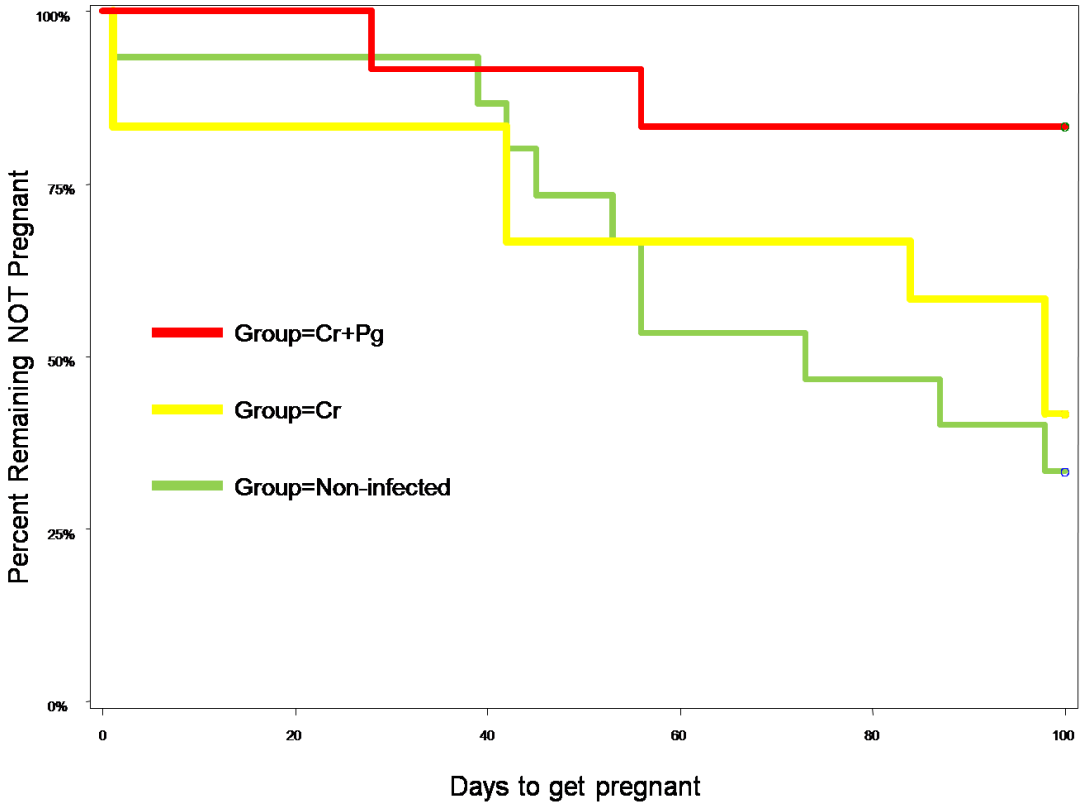
Averaged fetal weight values from infected mice were smaller when compared to those from non-infected mice. Columns and bars represent means and standard errors. Cr = *C. rectus*; Pg = *P. gingivalis*; *P < 0.05, ANOVA.

combined infection got pregnant. When cumulative pregnancies events were estimated under Kaplan Meier analysis, it was found that the *C. rectus* and *P. gingivalis* group had the lowest fecundity rate (Hazard Ratio 0.19[0.041-0.856], P=0.03) compared to *C. rectus* group (HR 0.87[0.310-2.154], P=0.68) and to non-infected mice (Figure 2.3).

Oral infection induced placental inflammation and TLR4 expression. Figure 4 presents representative histological findings on murine placentas. In the control placental tissues, there were scattered inflammatory cells present in the maternal decidua of some samples (not shown). In contrast, areas of focal necrosis and increased inflammatory cell infiltrate were apparent in placentas from infected mice (Figure 2.4A). Under confocal microscopy, the qualitatively analysis of murine placentas from infected mice showed enhanced TLR4 immunofluorescence, particularly more evident in labyrinth trophoblasts (Figures 2.4B-F).

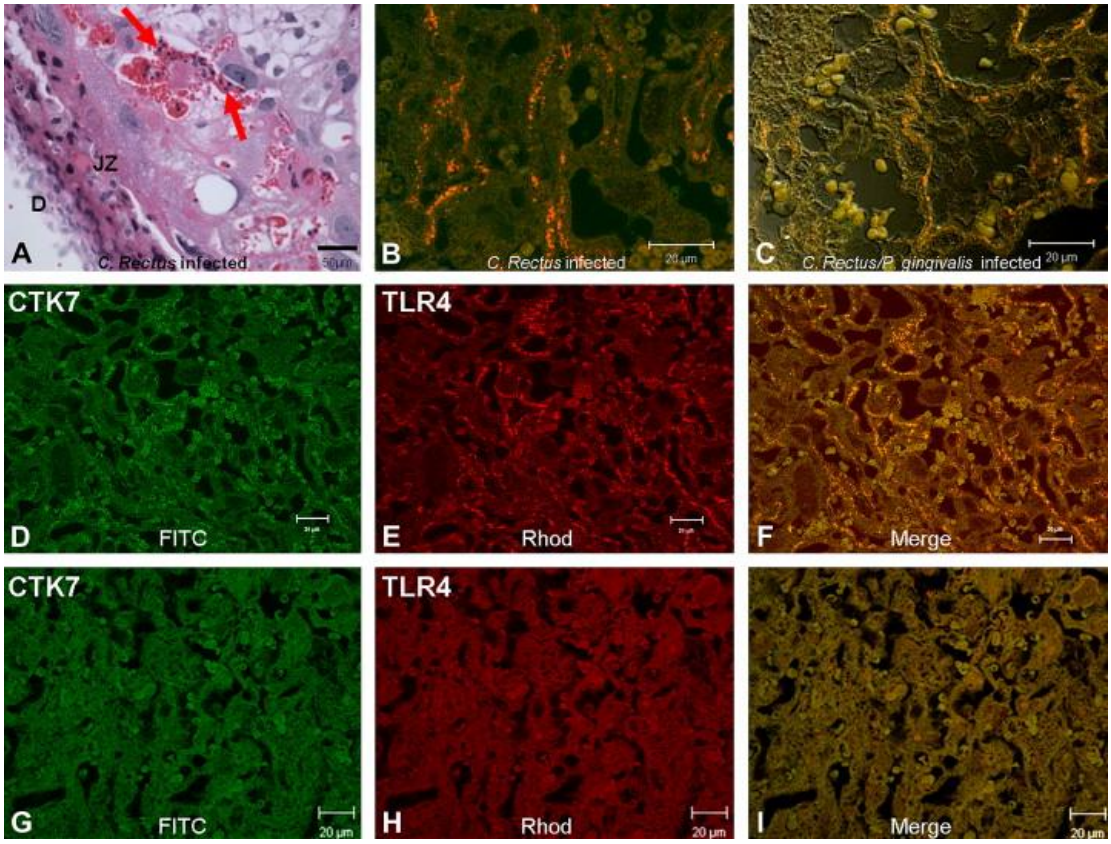
Oral infection increased placental TLR4 mRNA expression. In agreement with the histological observations, mRNA expression of TLR4 receptors was found to be significantly increased in the *C. rectus* group (1.98±0.886 fold difference, P<0.01 *ANOVA*) as shown in Figure 2.5. Even though the TLR4 expression was also higher in the *C. rectus* and *P. gingivalis* group, no significant differences were observed when compared to non-infected mice (1.29±0.871, P=0.06).

Figure 2.3 Oral infection affects murine fecundity.



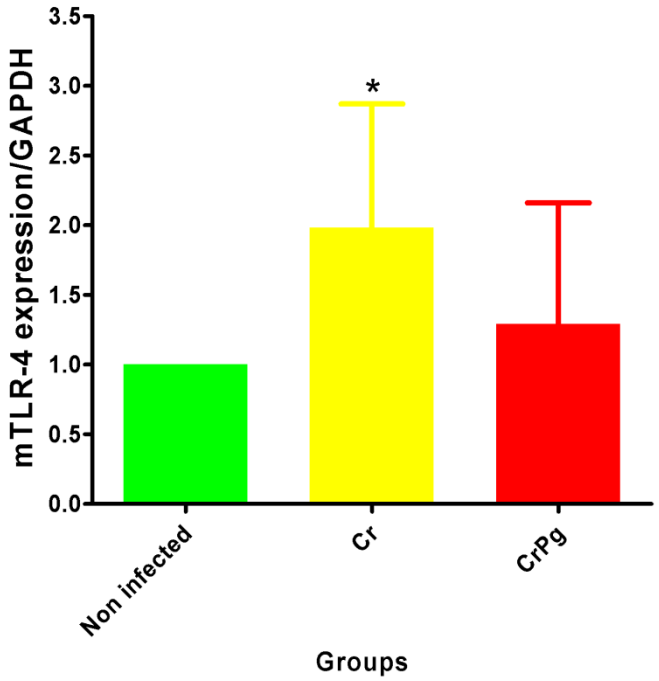
Kaplan–Meier analysis. *C. rectus* and *P. gingivalis* infected mice had the lowest fecundity rate (16.7%, HR 0.19[0.041–0.856], P = 0.03, Kaplan–Meier) compared to *C. rectus* (58.3%, hazard ratio 0.87[0.310–2.154], P = 0.68) and to non-infected mice.

Figure 2.4 Oral infection induced inflammation and TLR4 expression in murine placentas.



Histological and immunofluorescence analysis of murine placentas. A: Representative image (H&E stained) depicting extensive junctional zone (JZ) along with an increased inflammatory cell infiltrate (arrowheads) in placentas from infected mice. Decidua (D); bar = 50 μm. B and C: Immunofluorescence of a placenta from the *C. rectus* and *C. rectus/P. gingivalis* infected groups respectively. Images represent 3 merged channels (Rhodamine, FITC and DIC) depicting trophoblasts expressing TLR4 from both groups. D and E: Images depict the expression of Cytokeratin 7 (FITC-green) and TLR4 (Rhodamine-red) in labyrinth trophoblasts from a different *C. rectus*-infected section; F: Image illustrates co-localization of Cytokeratin 7 and TLR4 suggesting enhanced expression of TLR4 by labyrinth trophoblasts. G–I: Images correspond to fluorescence negative controls (no primary antibodies used) for Cytokeratin 7 and TLR4 respectively. Rhod = Rhodamine (red); FITC = fluorescein isothiocyanate (green); DIC = Differential Interference Contrast; white bars = 20 μm

Figure 2.5 Oral infection with periodontal pathogens increased TLR4 mRNA expression in murine placentas.



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

Discussion

This study sought to determine the effect of *C. rectus* and *C. rectus/P. gingivalis* oral infection on fetal growth restriction, fecundity and the placental TLR4 expression in a murine model of pregnancy. Previous experimental murine models of infection have examined the role of bacteria and/or isolated virulence factors (i.e. LPS) on preterm birth pathogenesis, reporting that C3HeB/FeJ mice develop up to 71% preterm births after heat-killed *E. coli* intrauterine injection (Hirsch & Wang 2005). However not many animal models of infection have addressed the pathogenic role of live oral bacterial infection on preterm births. Experiments with live periodontal bacteria have used subcutaneously-implanted stainless steel coiled chambers as an infection model to study a chronic distant infection in mice and rabbits, as it induces placental inflammation and growth restriction phenotypes (*C. rectus* and *P. gingivalis*) (Boggess et al. 2005a). The periodontal pathogen *Fusobacterium nucleatum* has also been used for intravenous injections in mice, showing subsequent placental inflammation and increased fetal resorptions and stillbirths (Han et al. 2004). Systemic bacterial dissemination of periodontal pathogens has been evidenced in blood, liver, uteri and individual placentas of growth-restricted fetuses (Lin et al. 2003b, Lin et al. 2003a, Bobetsis et al. 2007, Offenbacher et al. 2005, Yeo et al. 2005). This report provides evidence that the mixed oral infection model with *C. rectus* and *P. gingivalis* induces FGR and resorptions together with histological evidence of placental inflammation with areas of focal necrosis, enhanced TLR expression and impaired fecundity.

The oral infection model did not significantly correlate with FGR induction, conversely to our previous observations using the chamber model of infection (Yeo et al. 2005). This finding might be explained in part by the murine immune system ability to fight the oral infection, decreasing bacterial systemic dissemination and exposure protecting the developing fetuses. Particularly, Baker et al. reported that Balb/C mice are able to produce high titers of *P. gingivalis*-specific IgG using the same model of oral infection (Baker et al. 1994) and we have previously reported that fetal and placental growth may be unaffected in heat killed-*P. gingivalis* pre-immunized rabbits (Boggess et al. 2005b). In addition, the presence of *C. rectus* or *P. gingivalis* DNA was not detected in placentas coming from the oral infection model (data not shown). Nonetheless, overall infected groups had relatively less number of live fetuses, more resorptions and more growth restriction on average (Table 2.2 and Figure 2.2). Moreover, fecundity was significantly affected in infected groups (Figure 2.3). Only 16.7% of mice infected with *C. rectus* and *P. gingivalis* were able to get pregnant and had smaller litter sizes, situation that was not totally unanticipated as our previous observations on *P. gingivalis* infected animals suggested an impairment of fecundity [(Collins et al. 1994) and unpublished observations]. Therefore, we hypothesize that the difference in fetal restriction outcomes may be related to the mucosal immune system clearance at the portal of entry and the consequent low bacterial exposure at the placental level.

Even so, we found a placental inflammatory phenotype along with an enhanced TLR4 expression in murine placentas which is suggestive of an active

inflammatory response to bacterial exposure. Histologically, placentas from the infected groups showed apparent chronic inflammation (Figure 2.4A), and the double-staining immunofluorescence analysis showed that TLR4 expression was notably confined to trophoblasts in the labyrinth layer (Figures 2.4B-F). Furthermore, placental TLR4 mRNA levels showed to be significantly increased almost two-fold in the *C. rectus*-infected group and mildly increased (30%) in the double infection group approaching statistical significance (Figure 5, P=0.06). This finding might be explained by the limited number of placentas (n=12) available for analysis since only 17% (2 out of 12) mice were pregnant in the combined infection group.

TLRs are highly involved in responding to inflammatory processes in the presence or absence of infection in several tissues. In particular, TLRs are thought to be critical players of the innate immune response during pregnancy, which have significant implications for the success or failure in both early and late gestation (Gonzalez et al. 2007). Toll-like receptors expression has been described in the human placenta, mostly at the dominant cell type: the trophoblast (Abrahams & Mor 2005, Holmlund et al. 2002, Kumazaki et al. 2004). Trophoblasts are the first cells to differentiate from the outer layer of the blastocyst and are believed to participate during endometrial implantation and placental development (Cohen & Bischof 2007). Trophoblasts have also been proposed to coordinate the immune response during both processes by regulating regulate immune cells migration (macrophages and NK cells) to the endometrial implantation site through TLRs activation and chemokines production (Abrahams et al. 2005, Mor 2008, Mor et al. 2005). Furthermore,

abnormalities in decidual TLRs expression or function have been linked to abnormal placentation, inflammation, and adverse pregnancy outcomes (Krikun et al. 2007). Interestingly, our data suggest that murine placentas develop pro-inflammatory features after oral infection with periodontal pathogens, where placental trophoblasts notably express more TLR4 as compared to placentas from non-infected mice (Figures 2.4C-H), and these observations were also consistent with the placental mRNA levels (Figure 2.5). In fact, TLR4 receptors have been shown to mediate the murine placental inflammatory response and fetal death to *F. nucleatum* , and mice deficient for TLR-4 show protection against bacterial and LPS-induced preterm birth (Liu et al. 2007). Moreover, the selective antagonism of TLR-4 inhibits inflammation and preterm uterine contractility in a nonhuman LPS infection model in Rhesus monkeys (ms Waldorf et al. 2008).

The scope of this report was limited to determine TLR4 expression in response to oral Gram negative bacteria as proof of principle; however it is possible that other type of TLRs participate during the placental immune responses. For example, *C. rectus* offers a wide array of virulence factors including surface layer (S-layer) proteins (Thompson 2002), cytolethal distending toxin (CDT) (Braun et al. 1999), GroEL-like proteins (Hinode et al. 1998) and lipopolysaccharide (LPS)(Ogura et al. 1996). In addition, *P. gingivalis* also possess a plethora of virulence factors including capsule, fimbriae, proteases (Gingipains) and LPS (O'Brien-Simpson et al. 2004). Therefore, either *C. rectus* or *P. gingivalis* might be able to induce placental inflammation via different virulence factors that could potentially be sensed by

different TLRs including TLR-2 (peptidoglycans or fimbriae), TLR4 (LPS) or TLR-5 (flagellin).

In conclusion, previous models used to mimic chronic infections in mice result in dissemination of live oral bacteria to the placental tissues and impair fetal growth. However, this mixed oral infection model combines two commensal and critical pathogens that in combination are strongly associated with periodontal disease in humans (Offenbacher et al. 2007). Therefore, it is plausible to speculate that chronic systemic exposure to oral bacteria and related virulence factors may affect early pregnancy via pro-inflammatory mechanisms and such hypotheses warrant further investigations (Boggess et al. 2003). Further research is also needed to characterize the placental TLR response to oral pathogens in humans to elucidate and validate pathogenic mechanisms.

3. Chapter 3. Characterization of the invasive and inflammatory traits of oral *Campylobacter rectus* in a murine model of fetoplacental growth restriction and in trophoblastic cultures.

Abstract

Campylobacter species (*C. jejuni*, *C. fetus*) are well-established enteric abortofacients in humans and ungulates. *Campylobacter rectus* however is a periodontal pathogen that has recently been associated with human fetal exposure and adverse pregnancy outcomes including preterm delivery. Experimental evidence in pregnant mice has demonstrated that *C. rectus* can translocate from a distant site of infection to the placenta to induce fetal growth restriction and impair placental development. However, placental tissues from human, small-for-gestational age deliveries have not been reported to harbor *C. rectus* despite evidence of maternal infection and fetal exposure by fetal IgM response. This investigation examined the temporal relationship between the placental translocation of *C. rectus* and the effects on murine fetal growth. BALB/c mice were infected at gestational day E7.5 to examine placental translocation of *C. rectus* by immunohistology. *C. rectus* significantly decreased fetoplacental weight at E14.5 and at E16.5 ($P<0.05$). *C rectus* was detected in 63% of placentas at E14.5, but not at E16.5 ($P<0.005$). In addition, we studied the *in vitro* invasive potential of *C. rectus* in trophoblasts relative to *C. jejuni* by confocal immunofluorescence. *C. rectus* was able to effectively invade human trophoblasts (BeWo) in culture but not murine trophoblasts (SM9-1), and showed a trend for more invasiveness than that of *C. jejuni* ($P=0.08$). *C. rectus* challenge also significantly upregulated both mRNA and protein levels of human IL-6 and TNF-α in a dose-dependent manner in human trophoblasts (qPCR and ELISA), but did not increase cytokine expression in murine cells, suggesting a correlation

between invasion and cytokine activation. In conclusion, the trophoblast-invasive trait of *C. rectus* that appears limited to human trophoblasts may play a role in facilitating bacterial translocation and placental inflammation during early gestation.

Introduction

Campylobacter rectus is an exclusively oral Gram-negative, anaerobic and motile bacterium with a wide array of virulence factors including flagellum, surface layer proteins (S-layer), RTX-type toxins, GroELlike proteins and lipopolysaccharide (LPS) (Okuda et al. 1997, Wang et al. 2000, LaGier & Threadgill 2008). Together with other oral anaerobic bacteria, *C. rectus* is associated with the initiation and progression of periodontal disease (Ihara et al. 2003, Tanner et al. 1998, Socransky et al. 1998). *C. rectus* has been implicated in the association between periodontal disease and adverse pregnancy outcomes. For example, fetal exposure to *C. rectus* has been demonstrated to be higher in preterm than in full term neonates (Madianos et al. 2001). Moreover, *C. rectus* count levels are higher in the oral microbiota of pregnant women with increased salivary estradiol concentrations (Yokoyama et al. 2008). Indeed, *C. rectus* seems to thrive under high concentrations of estradiol and progesterone which have been shown to significantly enhance *C. rectus in vitro* growth (Yokoyama et al. 2005). Other *Campylobacter* spp. including *C. fetus* and *C. jejuni* have also been reported to be associated with miscarriages, premature labor and severe perinatal infection in both humans as well as in animals (Simor et al. 1986, Wong et al. 1990, Allos 2001, O'Sullivan et al. 1988b). It is then plausible that *C. rectus* may be an important contributor to adverse pregnancy outcomes due to its ability to disseminate systemically during pregnancy.

Our laboratory has studied the effects of *C. rectus* systemic infection on the fetoplacental unit using a murine model of intra-chamber injection with live bacteria

(Yeo et al. 2005). This intra-chamber model demonstrated that remote subcutaneous *C. rectus* maternal infection increases fetal resorptions and induces fetal growth restriction (Offenbacher et al. 2005). *C. rectus* infection also results in abnormal placental architecture, as evidenced by the decreased width of the vascular labyrinth and the increased width of decidual tissue in the placentas of infected growth-restricted mice (Bobetsis et al. 2007). If *C. rectus* disseminates systemically to reach the placenta it is then likely to interact with placental cells that express pattern recognition receptors (i.e. Toll-like receptors) (Abrahams et al. 2004), and subsequently induce a proinflammatory response that ultimately may contribute to an adverse pregnancy outcome. Indeed, recent results from our group have suggested that murine placentas from oral *C. rectus*-infected dams show enhanced placental TLR-4 expression along with increased vasodilation in the junctional zone surrounded by focal areas of inflammatory infiltrate (Arce et al. 2009).

The *in vitro* interactions of *C. rectus* with placental cells are yet to be studied. Hypothetically, direct *C. rectus* contact with trophoblasts may alter gene expression and induce a proinflammatory response. *C. rectus* may also have the ability to invade placental trophoblasts since other *Campylobacter* species have been shown to readily invade host or immunocompetent cells, a feature that may play a role in their virulence potential. For example, *C. jejuni* invasion of enterocytes has been shown to induce oncotic changes in these cells with extensive cytoplasmic vacuolation and loss of plasma membrane integrity, an important feature in the pathogenesis of bacterial enteritis (Kalischuk et al. 2007). Moreover, bacterial invasion into mammalian cells

has also been proposed as an important mechanism to evade phagocytic immune cells and allow systemic dissemination and bacterial translocation to different tissues (Li et al. 2008, Medina et al. 2003).

In this report we evaluated the presence of *C. rectus* in the placenta of pregnant mice that were infected subcutaneously with live bacteria. We also evaluated the *in vitro* ability of *C. rectus* to invade human as well as murine trophoblast cells, and whether *C. rectus* infection induces changes in two important proinflammatory genes at the messenger RNA and protein levels.

Materials and Methods

Mouse Model of *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/c 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. After one month of healing, females were mated overnight with males of the same strain. 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* or saline. Mice were then sacrificed at E14.5 and fetuses (n=15 from 3 non-infected dams and n=25 from 4 infected dams) and their respective placental tissues were collected. In preliminary experiments to establish the growth restriction model we collected weight data for fetoplacental units obtained from 27 non-infected dams and 32 infected dams sacrificed at E16.5. For histological analysis, placentas were collected and bisected sagitally then fixed in 4% paraformaldehyde and embedded in paraffin. Sections (6 μm) were stained using standard hematoxylin and eosin protocols and imaged using a Nikon Microphot-FXA Microscope equipped with a QImaging Micropublisher CCD camera. Morphometric measurements of the area occupied by each placental layer, namely decidua,

spongiotrophoblast layer and labyrinth, were conducted using the "Image J" software (http://rsb.info.nih.gov/ij/). For detection of *C. rectus* in placental tissues, placentas from 2 control mice (n=10) and 2 infected mice (n=11) from gestational day E14.5 were examined by immunostaining. Briefly, sections were de-paraffinized, re-hydrated in ethanol/H_2O washes and permeabilized by incubation in 0.2% Triton X in PBS. Slides were then incubated for 1 h in blocking buffer (5% BSA, 1% goat serum and 0.2% Triton-X in PBS) and then incubated overnight at 4ºC with a FITC-conjugated anti-*Campylobacter* antibody (Kirkegaard & Perry Labs, MD) and Texas Red-conjugated Phalloidin (Invitrogen, CA). Slides were washed several times in PBS and visualized with a Zeiss LSM5 Pascal Confocal Laser Scanning Microscope and 63X na 1.4 plan apo and 100X na 1.3 plan neofluar lenses. Images were captured using the LSM510 software. Images obtained from preliminary E16.5 experiments were included for comparison purposes.

Mammalian cell lines: The human trophoblast cell line BeWo (ATCC CCL-98) derived from a human choriocarcinoma (Pattillo & Gey 1968) and the mouse trophoblast cell line SM9-1 (a kind gift from JS Hunt, University of Kansas Medical Center, Kansas City, KS) derived from a gestational day 9 Swiss-Webster mouse placenta (Bowen & Hunt 1999) were used for invasion experiments and cytokine assays. The Madin-Darby canine kidney epithelial cell line MDCK (ATCC CCL-34) was used as a positive control for *C. jejuni* invasion as documented elsewhere (Wine et al. 2008). 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. SM9-1 cells were grown in RPMI-1640 supplemented with 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol and penicillin/streptomycin. Culture medium for MDCK cells was Eagle's Minimum Essential Medium with 10% FBS. All cells were grown in T-25 flasks (Corning, Life Sciences, MA) or onto cover slips placed at the bottom of a well in 24-well plates for invasion analysis. All cells were grown at 37°C in 10% CO_2.

Bacterial cultures: *Campylobacter rectus* strain 314 or *Campylobacter jejuni* strain ATCC 43457 were used for infection experiments. *C. rectus* 314 is an oral isolate from a periodontal disease patient. *C. rectus* was grown under anaerobic conditions at 37°C on Trypticase soy broth agar (Becton Dickinson and Company, Cockeysville, MD) supplemented with 0.2% yeast extract, 0.3% Phytone peptone, 0.2% NaCl, 0.3% ammonium formate, 0.4% sodium fumarate and 0.4% L-asparagine, adjusted to pH 7.8. *C. jejuni* was grown on Mueller Hinton agar (Oxoid, Cambridge, UK) plates at 37°C under a microaerophilic atmosphere.

Invasion assays: Mammalian cells were grown onto cover slips in 24 well plates until 80-90% confluency. Bacterial strains were harvested from agar plates after 72 h incubation (48 h incubation yielded adequate growth for *C. jejuni*) and resuspended in tissue culture medium without antibiotics to an optical density of 1.00 (600 nm). Mammalian 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 100 bacteria/mammalian cell, after which plates were centrifuged at 250 × *g* for 5 min, incubated for 5h at 37°C in

10% CO_2 and washed with PBS. This time point and MOI were chosen for quantification as pilot experiments showed that a similar number of *C. rectus* cells were found intracellularly at 5h, 12h and 24h (data not shown). For visualization of adhesion/invasion, infected and non-infected controls were then washed 3 times with PBS to remove non-adhered bacteria and fixed with 4% paraformaldehyde for 30 min at room temperature. After washing twice with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 15 min. Blocking buffer (5% BSA, 1% goat serum and 0.2% Triton-X-100) was added for 1 h and cells were incubated overnight at 4°C with a FITC-conjugated anti-*Campylobacter* antibody and Texas Red-conjugated Phalloidin. Cells were then washed several times with PBS. Cover slips were mounted on glass slides and observed using a Zeiss LSM5 Pascal Confocal Laser Scanning Microscope and 63X na 1.4 planapo and 100X na 1.3 plan neofluar lenses. Random fields were captured using the LSM510 software. Quantification of bacterial adhesion and invasion was done manually using the LSM510 software and the ortho view to navigate the entire stack of images visualizing x, y and z planes. Image stacks of up to 8 random fields were used for quantification of invasion/adhesion by confocal microscopy.

Proinflammatory gene expression and cytokine assays: Infected cells at MOI 50, 100 and 500 during 12 hours and non-infected controls were washed 3 times with PBS to remove non-adhered cells. The MOIs were determined by performing preliminary experiments testing over a range of MOI that extended from 5 to 1000 MOI. Additional experiments were also performed to include ultrapure *E. coli* LPS

(0111:B4 strain, Invivogen, San Diego, CA) using 1ug/well as positive control for proinflammatory gene expression. Total RNA was isolated from cells with the use of the miRNeasy Mini Kit (Qiagen, CA). 1 µg of total RNA was reversely transcribed using the Omniscript system (Qiagen, CA). Real-time PCR was performed with 1 µL of the cDNA reaction in a 7500 Sequence Detection System (ABI Prism, Applied Biosystems, CA). TaqMan pre-inventoried assays for human (Hs00985639_m1) and mouse (Mm00446190_m1) Interleukin 6 (IL-6) as well as for human (Hs99999043_m1) and mouse (Mm99999068_m1) tumor necrosis factor alpha (TNF-α) were used for relative gene expression quantification. Reactions were performed in duplicates and two independent assays were carried out for each gene. The 18S ribosomal RNA subunit gene and the Glyceraldehyde-3-Phosphate Dehydrogenase (gapdh) gene were used as endogenous housekeeping controls for human (BeWo) and murine (SM9-1) trophoblast cells respectively. Results were evaluated using the delta-delta Ct method, where delta Ct was calculated as (gene of interest Ct) − (housekeeping gene Ct), and the relative quantity of mRNA expression was calculated by the delta–delta Ct as $2^{-[(\text{infected sample delta Ct})-(\text{non-infected sample delta Ct})]}$. The quantification of IL-6 and TNF-α in cell supernatants was performed by means of xMAP multiplexing cytokine assays. Briefly, cell supernatants were collected after timed infection, centrifuged at 1.500 x g for 5 minutes and then frozen until analysis. Multianalyte kits for human and mouse IL-6 and TNF-α were used following the manufacturer instructions (Fluorokine MAP Kits, R&D systems, MN).

Statistical analysis: A minimal sample size of 8 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 (Yeo et al. 2005). Continuous variables were expressed as means and standard deviations. Mean placental weight values for control and infected mice were compared using the unpaired Student's t test. The detection frequency of *C. rectus* in infected and non-infected murine placentas was compared by using the Fisher's exact test. Counts of *C. rectus* invasion/adhesion in cells were compared using the unpaired Student's t test. mRNA fold differences and protein concentration differences were compared using one-way ANOVA. The threshold for statistical significance was set at a P value less than 0.05. All analyses were performed using SAS v.9.2. (SAS Institute, Cary, NC).

Results

***C. rectus* induces growth restriction and translocates from a distant site of infection to the murine placenta *in vivo*.** *C. rectus* infection impaired the development of the fetoplacental unit at day E14.5 and E16.5 (Table 3.1). At E14.5, mice infected with *C. rectus* had a significant lighter placental weight (0.104±0.02 grams) and fetoplacental weight (0.236±0.04 grams, n=25) compared to the average placental weight (0.118±0.02 grams, P=0.03) and fetoplacental weight (0.262±0.03, n=15, P=0.02) of non-infected mice. At E16.5, fetoplacental weight showed a similar trend to that of E14.5 (0.570±0.004 grams in *C. rectus*-infected mice vs. 0.612±0.004 grams in non-infected mice) and this difference was even more significant (P>0.0001); however we observed a different trend for placental weight at E16.5, where *C. rectus*-infected placentas were heavier than non-infected controls [0.14±0.005 vs. 0.132±0.002 grams respectively, (P>0.0001)], probably because infected placentas presented higher cellular inflammatory infiltration leading to placental edema (Offenbacher et al, 2005). Also, in contrast to our previous findings at E16.5 (Bobetsis et al. 2007), the morphometric analysis of the placentas from infected mice showed no significant differences in the mean percentage area of each of the placental zones when compared to controls at E14.5 [49±8.45 vs. 53.8±14.0 respectively, P=0.08); however, a trend for an increase in the size of the decidua layer in infected placentas from fetuses with the lowest weight was noted. We also observed focal areas of inflammatory infiltrate in the junctional zone of infected placentas as reported previously for E16.5 (data not shown).

Table 3-1 Growth restriction in fetoplacental units at day E14.5 and E16.5 after *C. rectus* infection.

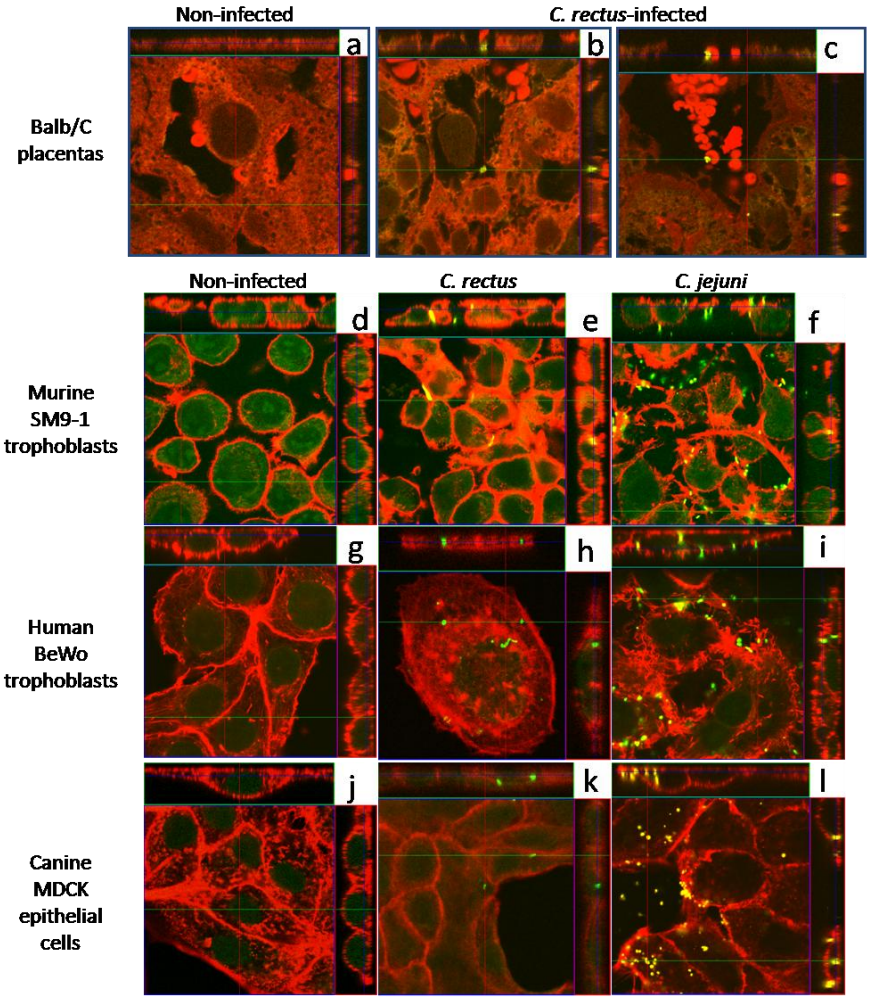
Gestational Day / Weight		Non-infected Control	*C. rectus*-infected	P value
E14.5	Placental weight	0.118±0.020 *(n=15)*	0.104±0.020 *(n=25)*	**0.03**
	Fetoplacental weight	0.262±0.030 *(n=15)*	0.236±0.040 *(n=25)*	**0.02**
E16.5	Placental weight	0.132±0.002 *(n=143)*	0.14±0.005 *(n=105)*	**<0.0001**
	Fetoplacental weight	0.612±0.004 *(n=143)*	0.570±0.004 *(n=105)*	**<0.0001**

Data represent mean values ± SD per group. Pregnant mice received an intra-chamber injection of live *C. rectus* or saline at E7.5. Mice were sacrificed at E14.5 and 16.5 for the analysis of their fetoplacental tissues. Mean placental and fetoplacental weight were significantly different between non-infected controls and *C. rectus*-infected mice. n values correspond to the number of fetuses or placentas analyzed.

Immunofluorescence labeling of *C rectus* on murine placental tissues and trophoblasts in culture is depicted in Fig. 3.1. *C. rectus* was detected in 7 out of 11 stained placentas (63.6%) from infected mice (Fig. 3.1b and 3.1c) and in none of the 10 placentas from non-infected controls (Figure 3.1a) (P=0.004) at E14.5. The presence of bacterial cells was not observed in the majority of the scanned fields in infected placental tissues, as the bacteria were rather scarcely distributed and seen as single or coupled bacteria cells. No bacterial cells were observed in murine placentas analyzed at E16.5 (data not shown).

***C. rectus* adheres and invades human trophoblasts.** *C. rectus* adhesion and invasion, as visualized by confocal microscopy, was evaluated in murine and human trophoblasts. In addition, we compared the invasiveness of the oral *C. rectus* to a highly invasive intestinal pathogenic strain of *C. jejuni*. Epithelial cells (MDCK) served as positive control for Campylobacter invasion. Fig.3.1e shows extracellular and intercellular localization of *C. rectus* without cytoplasmic invasion in murine trophoblasts. At the same microbial load, there was much greater accumulation of *C. jejuni* extracellularly (Fig. 3.1f) without appreciable evidence of intracellular invasion for either *C. jejuni* or *C. rectus*. However, both bacteria were able to penetrate cell junctions and *C. jejuni* was more efficient than *C. rectus* at doing so (Table 3.2). In contrast, both *C. rectus* and *C. jejuni* were able to invade and localize intracellularly in the human trophoblast BeWo cells, as well as in the positive control MDCK cells (Figure 3.1g-l). In all cases, *C. jejuni* adhered, penetrated through cell junctions and invaded with significantly more efficiency than *C. rectus*, except for intracellular

Figure 3.1 *C. rectus* invasiveness in murine placentas and mammalian trophoblasts.



Immunofluorescence analysis of murine placentas: (a–c) representative images in murine placentas from non-infected (a) and infected mice (b and c). C. rectus was seen in placentas of infected mice (64%) but not in the placentas of control mice. The presence of bacterial cells was rather scarce and was not observed in the majority of the scanned fields: (d–i) representative images from in vitro trophoblastic cell infection experiments and non-infected controls. Bacteria were found extracellularly attached to murine trophoblasts, or present in the cell junctions within cells (e) without apparent evidence of cytoplasmic invasion (f). Conversely, *C. rectus* and *C. jejuni* were detected intracellularly in the human trophoblast BeWo cells (h and i). (j–l) The canine epithelial MDCK cells served as positive control target cells using a highly invasive intestinal pathogenic strain of *C. jejuni*. For all image stacks, red stain corresponds to F-actin stained with Texas Red-conjugated Phalloidin. Green-yellow fluorescent cells represent bacteria stained with an FITC-conjugated Campylobacter-specific antibody. Magnifications: [a–c] at 63× na 1.4 plan apo and [d–l] at 100× na 1.3 plan neo fluar lenses.

Table 3-2 Quantification of adhesion and intercellular/intracellular localization of *C. rectus* and *C. jejuni* in trophoblast cells *in vitro*.

Cell line	Bacteria	Bacteria adhered/cell	Bacteria colocalizing with actin (cell junctions) per cell	Invasive bacteria/cell (cytoplasmic)
SM9-1	*C. rectus* 314	0.50±0.41 *	0.44±0.10 *	0.00±0.00
	C. jejuni 43457	4.79±2.82	1.53±0.71	0.00±0.00
BEWO	*C. rectus* 314	0.43±0.52 *	0.08±0.11 **	5.51±3.18
	C. jejuni 43457	5.30±1.75	2.83±0.32	2.96±0.77
MDCK	*C. rectus* 314	0.26±0.36 *	0.10±0.12 *	0.69±0.25 *
	C. jejuni 43457	3.83±2.40	1.40±0.65	1.84±1.19

Data represent the mean number of bacterial cells counted in the entire stack of images in up to 8 random fields per sample (Fig. 3.1). *C. jejuni* and *C. rectus* adhered to SM9-1 murine trophoblast cells but invasion was negligible. In contrast, both *C. jejuni* and *C. rectus* invaded the human trophoblast cell line BeWo and the canine epithelial cell MDCK ($P < 0.05$). It was noted that in most cases *C. jejuni* adhesion/invasion of cells was significantly greater than that of *C. rectus*. However, *C. rectus* showed a non-significant trend of higher invasiveness counts in BeWo cells when compared to *C. jejuni* ($P = 0.08$). *$P < 0.05$ and **$P < 0.01$.

invasion of the human trophoblast cell line (BeWo) in which *C. rectus* showed a non statistically significant trend for greater invasion over *C. jejuni* (P=0.08).

***C. rectus* upregulates IL-6 and TNFα gene expression in human trophoblasts.** Table 3.3 illustrates the mRNA relative gene fold differences of *C. rectus*-infected cells vs. non-infected controls. Human BeWo cell responses to the positive control (*E. coli* LPS) were upregulated for TNF-α (67.63±0.65) and IL-6 (11.47±0.28); however gene expression responses to LPS were particularly low for the murine SM9-1 cells for both IL-6 (0.74±0.28) and TNF-α (1.12±0.34). A statistically significant trend was also observed in *C. rectus*-infected cells, in which dose-dependent responses of proinflammatory genes were observed for IL-6 ($P<0.05$) and TNF-α ($P<0.001$) in human BeWo cells, but conversely relative gene differences in the murine SM9-1 cells were low for IL-6 and TNF-α (Table 3.3).

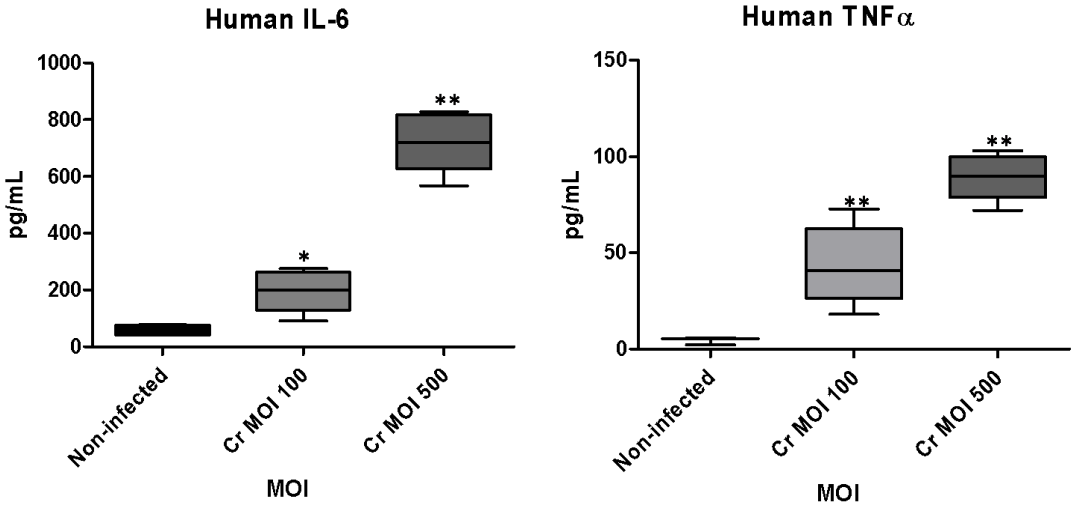
***In vitro C. rectus* infection upregulates proinflammatory cytokine expression in human trophoblasts.** Figure 3.2 depicts the cytokine concentration differences in cell supernatants from infection experiments at 12 hrs. Both human IL-6 and TNF-α were increased in a MOI-dependent manner. Protein concentration differences were significantly different for human IL-6 ($P<0.0001$) and TNF-α ($P<0.0001$). Murine proinflammatory cytokines were not detected in cell supernatants after infection with *C. rectus*.

Table 3-3 Relative proinflammatory gene expression in trophoblastic cultures after *C. rectus* infection *in vitro*.

Gene	MOI	mRNA expression fold differences
Human *IL6*	50	2.75 ± 0.38
	100	4.18 ± 0.85*
	500	6.39 ± 0.61**
Human *TNFα*	50	10.9 ± 0.54**
	100	24.6 ± 0.55**
	500	56.4 ± 1.96**
Murine *IL6*	50	0.5 ± 6.88
	100	4.15 ± 1.87
	500	2.31 ± 2.10
Murine *TNFα*	50	1.23 ± 0.64
	100	1.0 ± 0.34
	500	1.24 ± 0.49

Data represent mean fold changes ± SD normalized to non-infected controls. Cells were infected at MOI 50, 100 and 500 during 12 h and the relative expression of human or murine IL-6 and TNFα was quantified by qPCR.

Figure 3.2 Proinflammatory cytokine expression in human trophoblasts after *in vitro C. rectus* infection.



Box plots depict mean ± SD cytokine concentration values. Cell supernatants were harvested and cytokines were quantified by xMAP multiplexing. Human IL-6 and TNFα were significantly increased in a MOI-dependent manner. *P < 0.05 and **P < 0.0001.

Discussion

The chamber infection murine model used in this report aims to simulate bacterial chronic exposure in the murine host mimicking the repeated systemic challenge that would be associated with a chronic oral infection. Here we report fetoplacental unit restriction at gestational day E14.5 (Table 3.1) along with evidence of *C. rectus* translocation/invasion to the murine placenta *in vivo*. The human clinical isolate *C. rectus* 314 was detected in 64% of murine placentas after 7 days post infection at E14.5 by immunohistology (Fig. 3.1a-c), but *C. rectus* was not detected at E16.5 using the same technique (data not shown). Interestingly, this finding could mean that intact bacteria can be found in the placenta at E14.5 but only bacterial DNA is detected at E16.5 by means of nested PCR amplifications (Bobetsis et al. 2007). Since immunofluorescence not only detects viable bacteria but also dead bacterial cells, such differences may be partially explained by *C. rectus* losing viability due to limited growth conditions or due to immune clearance. Interestingly, *C. rectus* detection in placental tissues from human, small-for-gestational age deliveries have not been reported yet, despite evidence of a remarkable degree of fetal antibody seropositivity (cord blood IgM) to *C. rectus* (Madianos et al. 2001).

Host cell invasion is an important feature in the pathogenesis of many bacteria, including members of the Campylobacteraceae family. *Campylobacter* invasion into host cells has been studied mainly with the enteric pathogen *C. jejuni* (Fields & Thompson 2008, Krause-Gruszczynska et al. 2007). The invasiveness of *C. jejuni* in cultured epithelial cell lines has been proposed to correlate with colonization

efficiency and disease outcome (Bacon et al. 2000, Hanel et al. 2004). However, to the best of our knowledge this investigation represents the first study to present evidence of *in vitro C. rectus* invasion in trophoblastic cells and this invasion process appears to be specific for human trophoblasts (Fig. 3.1). The invasiveness of *C. rectus* evaluated in this study via confocal microscopy appears to be within the range reported for other Campylobacter spp. in terms of quantity (0.01-2 bacteria per cell) (Biswas et al. 2004). In these studies the number of internalized bacteria seems to increase with greater concentrations reaching a plateau at a MOI of 100-200; however, even with the most invasive strains only one to three bacteria are internalized per cell (Friis et al. 2005). Even though *C. jejuni* showed significantly greater activity in terms of cellular adhesion and paracellular translocation, this study supports the finding that scarce bacterial numbers of *C. rectus* or *C. jejuni* get internalized in human BeWo trophoblasts using similar MOIs. However, we found that *C. rectus* preferentially invades human trophoblasts *in vitro* (Table 3.2), which may indicate that *C. rectus* is specifically targeting the human placental trophoblast which may contribute to the apparent fetoplacental tropism proposed in humans (Madianos et al. 2001). Furthermore, the invasion efficiency of *C. rectus* appeared to be greater than the invasiveness of an established placental pathogen such as *C. jejuni*, at least for the *C. jejuni* strain tested. Similar routes of infection from mucosal surfaces to other tissues have been described for different *C. jejuni* strains, which have been shown to internalize into host cells localizing intracellularly (van Alphen et al. 2008) and also to translocate through cell junctions disrupting tight junction

protein components (Chen et al. 2006). Either bacterial paracellular (through junctions between cells) translocation or transcellular (via the cell cytoplasm) internalization could potentially be useful for *C. rectus* to cross the oral epithelial barrier and to migrate to distant sites including the developing fetoplacental unit. The ability to invade human trophoblasts and localize in their cytoplasm may also confer *C. rectus* protection from immune surveillance while in the immunologically-protected placental tissue, as demonstrated in other abortofacients such as *Listeria monocytogenes* (Bakardjiev et al. 2006).

This study also demonstrated that in response to *C. rectus* challenge a pro-inflammatory response is induced in human trophoblastic cells. A clear dose-dependent (MOI) proinflammatory activity was observed in the human BeWo cell line (Table 3.3 and Fig. 3.2) as evidenced by the gene expression and protein upregulation of IL-6 and TNF-α, two important members of the cluster of proinflammatory cytokines involved in pathogenesis of chorioamnionitis, preterm labor, low birth weight and preterm premature rupture of membranes associated with intraamniotic infections (Gomez et al. 1997). Furthermore, among different placental cell populations, trophoblasts in particular have been proposed to play a key role in coordinating placental immune responses during implantation and pregnancy maintenance (Mor 2008). Trophoblasts are also thought to be primary sentinel cells that are involved in microbial clearance preventing microbial translocation of infectious agents from mother to fetus (Levy 2007b).

The results obtained with human trophoblasts contrast to those obtained with murine trophoblasts, which were unresponsive to the *C. rectus in vitro* challenge and did not show significant proinflammatory activity (Table 3.3). Even though the SM9-1 cell line has been reported to express TNF-α mRNA (Sharma 1998), these murine trophoblasts derived from Swiss mice did not offer a significant proinflammatory response when challenged by *C. rectus*. This finding is also supported by the observation that SM9-1 murine trophoblasts were not responsive to *E. coli* LPS-challenge (1ug/mL) although cells remained viable based upon proliferation assays (data not shown). Moreover, *C. rectus* did not appear invasive in the mouse trophoblast cultures, but did co-localize at regions of intercellular junctions suggesting that this human pathogen may use a paracellular pathway to effect translocation to promote murine growth restriction. In fact, we have observed the bacteria confined to blood vessels in the labyrinth rather than in the placental tissues, suggesting a different invasion mechanism *in vivo.* Similarly, it is known that differences exist in the response of human and murine epithelial cells to other *Campylobacter* species, as evidenced by differences in the expression of genes involved in growth, transcription and steroid biosynthesis in human colonic epithelia vs. unresponsive murine colonic cells (Rinella et al. 2006). Others have also reported that *C. jejuni*, which normally does not bind to Chinese hamster ovary cells, binds avidly when these cells are transfected with the human alpha1,2-fucosyltransferase gene, thus further demonstrating the specificity of *Campylobacter* spp. for human cells (Ruiz-Palacios et al. 2003). To summarize, the differences between human and

murine responses suggest that *C. rectus* may have a recognition system that is specific for human trophoblasts which could potentially enhance tropism and immune activation in the human host.

In conclusion, our studies demonstrated that *C. rectus* has the ability to translocate *in vivo* from a distant site of infection to the fetoplacental unit. The ability of *C. rectus* to invade human trophoblast cell lines and localize intracellularly as well as its ability to increase the expression of important proinflammatory cytokines highlights its potential to be an important contributor to adverse pregnancy outcomes associated with periodontal disease in humans.

4. Chapter 4. TLR4 mediate intrauterine growth restriction after systemic *C. rectus* infection.

Abstract

Campylobacter rectus is a periodontopathogen associated with fetal exposure and increased risk for low-birth weight and prematurity in humans. In pregnant mice, *C. rectus* invades placental tissues and subsequentially induces both local placental inflammation and fetal intrauterine growth restriction (IUGR). Placental type-4 Toll-like (TLR4) receptors expression has been reported to be upregulated after *C. rectus* infection, but it is still unclear whether TLR4 mediates placental inflammatory responses and IUGR onset *in vivo*. The objective of this investigation was twofold: to examine the effect of *in vitro* TLR4 neutralization in trophoblastic pro-inflammatory activity and to study the IUGR phenotype in a congenic TLR4-mutant mouse strain after systemic *C. rectus infection in vivo*. BeWo trophoblastic cells were infected with *C. rectus* and the subsequent gene expression was assessed by means of a TLR-specific pathway superarray. Pro-inflammatory cytokine production was assessed by means of a multiplexing assay. A subcutaneous chamber model was used in BALB/cAnPt ($TLR4^{Lps\text{-}d}$) and BALB/cByJ wild-type (WT) females (N=6/group). Mice were mated overnight (12H) with corresponding WT or TLR4 deficient males once/week and monitored for vaginal plugs/weight changes. Pregnant females were then infected (or sham infected) at gestational day (E)7.5 and sacrificed at E16.5 to assess IUGR by means of fetal weight/length measurements (grams±SE or centimeters±SE). Homozygous ($TLR4^{lps\text{-}d\text{-}/\text{-}}$) and Heterozygous ($TLR4^{lps\text{-}d+/\text{-}}$) fetal phenotypes were selected for analysis. Groups were compared to sham-infected BALB/cByJ (WT) mice using the same model (N=27). 143, 37, 47 and 49 fetuses

served as unit analysis from sham-infected WT, *C. rectus*-infected WT, *C. rectus*-infected TLR4$^{lps\text{-}d\text{-}/\text{-}}$ and *C.rectus*-TLR4$^{lps\text{-}d+/\text{-}}$ groups respectively. We found that the TLR4 gene is differentially expressed in *C. rectus*-infected human trophoblasts over other TLRs along with other important pro-inflammatory genes. Neutralizing anti-human TLR4 antibodies significantly impaired the production of pro-inflammatory cytokines in BeWo cells after *C. rectus* infection in a dose-dependent manner. Litter size/resorptions frequencies among groups showed no statistical differences ($P>0.05$, Chi-square). Maternal *C. rectus* infection significantly decreased fetal weight (0.42 ± 0.057 vs. 0.48 ± 0.068, $P<0.05$ Anova) and fetal length (1.23 ± 0.094 vs. 1.44 ± 0.100, $P<0.05$) in infected WT when compared to sham WT controls. However, infected TLR4$^{lps\text{-}d\text{-}/\text{-}}$ mice did not show statistically significant differences in fetal weight (0.46 ± 0.073, $P>0.05$) and length (1.40 ± 0.119, $P>0.05$) when compared to WT controls. Infected WT and infected TLR4$^{lps\text{-}d\text{-}/\text{-}}$ showed to be significantly different in their IUGR phenotype ($P<0.05$, Anova). Furthermore, Heterozygous TLR4$^{lps\text{-}d+/\text{-}}$ fetuses showed IUGR phenotype again and were not statistically different from the infected WT mice ($P>0.05$). In conclusion, our data indicates that TLR4 receptors are important in mediating pro-inflammatory responses at the trophoblastic cellular level and TLR4-deficient fetuses seem to be less susceptible to develop IUGR in response to maternal *C. rectus* infection when compared to sham-infected WT mice, suggesting that placental proinflammatory cytokine activation is likely to be mediated by TLR4 during low birth weight/preterm delivery pathogenesis.

Introduction

Periodontal diseases (gingivitis and periodontitis) are part of 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 diabetes (Beck & Offenbacher 2005). Periodontitis has also been associated to an increased risk for preterm delivery (PTD) and preeclampsia in different human populations, suggesting that maternal periodontitis and associated bacteria represent an important systemic stressor to both the mother and fetus (Ruma et al. 2008). Even though vaginal/uterine microbial infections are recognized to be the major contributors to PTD pathogenesis, other pathogens have shown coming from different focal infections in the body and reaching the developing fetus through hematogenous dissemination. In particular, *Campylobacter rectus* is an exclusively oral Gram negative anaerobe harbored in periodontitis-associated oral biofilms 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. Furthermore, our clinical studies have found that maternal *C. rectus* fetal exposure is more frequent in preterm delivery cases, as evidenced by low maternal IgG and high fetal IgM antibody responses

(Madianos et al. 2001). However, the underlying biological mechanisms leading to preterm delivery after oral *C. rectus* infection 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 and is associated with low birth weight (<2500 grams) and long-term adverse sequelae (cerebral palsy, hearing and learning problems)(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). Even though the precise triggering mechanism has not been established, the development of a proinflammatory condition is a common pathway that centralizes all multiple risk factors(Romero et al. 1994). Particularly, uterine infections account for 25–40% of preterm births and they are strongly linked with the inflammatory pathway onset. Uterine infections are known to upregulate the production of local pro-inflammatory cytokines, metalloproteinases and prostaglandins that lead to membrane weakening, early membrane rupture and uterine contraction initiation(Shoji et al. 2007). If inflammation takes place in all multiple PTD-associated risk factors, then proinflammatory responses are likely to be initiated/mediated by the host innate immune system. Toll-like receptors (TLRs) are a family of transmembrane proteins that have a primary role in pathogen recognition and innate immunity initiation (Brikos & O'Neill 2008). TLRs receptors bind to several microbial components or end-products known as pathogen-associated

molecular patterns (PAMPs), which include peptidoglycans, lipoteichoic acid, flagellin, double-stranded viral RNA, unmethylated bacterial DNA and lipopolysaccharide (LPS) among others. 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 & 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). TLRs are highly involved in responding to inflammatory processes in the presence or absence of infection. In particular, TLRs are thought to be a critical component of the innate immune response during pregnancy, which have significant implications for the success or failure of pregnancies in both early and late gestation (Patni et al. 2007). To date, 13 mammalian Toll-like receptors homologues have been identified and designated, and their expression has been described in the human placenta, mostly at the dominant cell type: the trophoblast (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). For example, trophoblast are believed to regulate immune cells migration (macrophages and NK cells) to the endometrial implantation site by means of TLRs activation and subsequent chemokines production (Abrahams et al. 2005); abnormalities in decidual TLRs

expression or function have been linked to abnormal placentation, inflammation, and adverse pregnancy outcomes (Krikun et al. 2007).

Our lab has worked on understanding the maternal and fetal biological mechanisms leading to preterm delivery in response to *C. rectus* infection. We have hypothesized that *C rectus* induces a placental innate inflammatory response mediated by Toll-like receptors (TLRs). This hypothesis has been elaborated based on our observations in pregnant mice experiments using *C. rectus* as a model of systemic infection, in which we have demonstrated: 1) Systemic dissemination ability 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 smaller pups) (Yeo et al. 2005); 4) Altered gene expression along with imprinted genes down-regulation (i.e. Insulin growth-factor 2) via changes in DNA methylation patterns (hypermethylation) (Bobetsis et al. 2007, Bobetsis et al. 2010); 5) trophoblastic TLR-4 increased expression 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 on 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 mediates placental inflammatory responses and IUGR onset in vivo in response to *C. rectus* exposure. Therefore, this investigation first examined the expression patterns

of several genes associated with the TLR pathway including different TLRs by means of a gene expression array from *C. rectus* infected trophoblastic cells *in vitro*. Then we evaluated the effect of using TLR4 neutralizing antibodies in the trophoblastic inflammatory response measured by the production of pro-inflammatory cytokines. Finally, we evaluated the *in vivo* IUGR phenotype in a congenic TLR4-mutant mouse strain on a Balb/C background after systemic *C. rectus* infection.

Materials and Methods

Mammalian cell lines: The human trophoblast cell line BeWo (ATCC CCL-98) derived from a human choriocarcinoma (Pattillo & Gey 1968) was used for gene expression analysis and cytokine assays. 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 $1x10^9$ bacteria/ml.

***In vitro* 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 done to evaluate the effect of using a TLR4 neutralizing antibody in the previously observed pro-inflammatory responses. 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 assays and TLR pathway-specific gene expression experiments: The quantification of IL-6 and TNF-α in cell supernatants was performed by means of xMAP multiplexing cytokine assays. Briefly, cell supernatants were collected after timed infection, centrifuged at 1.500 x g for 5 minutes and then frozen until analysis. Multianalyte kits for human IL-6 and TNF-α were used following the manufacturer instructions (Fluorokine MAP Kits, R&D systems, MN). Infected cells and non-infected controls were washed 3 times with PBS to remove non-adherent cells. Cells

were detached using trizol and total RNA was isolated from cells with the use of the miRNeasy Mini Kit (Qiagen, CA). 1 µg of total RNA was reversely transcribed using the RT^2 first strand kit (C-03, Qiagen, CA). Real-time PCR was performed with a total of 1ug of the cDNA reaction in a RT² Profiler™ PCR Array Human specific for Toll-Like Receptor Signaling Pathway (PAHS-018A, Qiagen, CA). This platform was used to evaluate gene expression changes in infected vs. non infected (control) BeWo trophoblastic cells. Reactions were performed using 3 biological replicates per group. The Glyceraldehyde-3-Phosphate Dehydrogenase (gapdh) and the Actin-B genes were used as endogenous housekeeping controls. Results were evaluated using the delta-delta Ct method available at the manufacturer's web-based PCR array data analysis software. All plates were processed in a 7500 Sequence Detection System (ABI Prism, Applied Biosystems, CA).

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($TLR4^{Lps-d}$) mice and BALB/cByJ corresponding Wild-type (WT) controls were bought from the Jackson Laboratory (C.C3-$Tlr4^{Lps-d}$/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 $TLR4^{lps\text{-}d\text{-}/\text{-}}$ infected dams and n=49 from heterozygous $TLR4^{lps\text{-}d+/\text{-}}$ infected dams) and their respective placental tissues were collected for further analyses (fetal length/weight). For comparison purposes, we also included data from previous experiments using sham-infected WT controls (n=143) and *C. rectus*-infected WT mice (n=37) that were part of the same experimental animal model.

Statistical analysis: A minimal sample size of 6 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. 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. All analyses were performed using GraphPad software (San Diego California USA).

Results

TLR4 gene is differentially expressed in *C. rectus*-infected human trophoblasts over other TLRs. We first evaluated the expression differences of 80 selected mRNA genes in a TLR pathway-specific array platform after *in vitro C. rectus* (test) or sham (control) *in vitro* infections in trophoblastic BeWo cells. Table 4.1 depicts the gene table results for fold differences, fold up/down regulation and T-test P values. Overall, most genes (72 genes, 90%) showed a trend towards down-regulation when comparing test/control experiments. Among these, only 8 genes (11%) were statistically significant ($P<0.05$). In terms of up-regulated genes (8 genes, 10%), only 2 genes (25%) were statistically significant (Caspase 8 and Tumor necrosis factor receptor 1A). Among up-regulated genes, important members of the TLR pathway such as Caspase 8, Colony stimulating factor 2, Interleukin 2, Interleukin 6 and Tumor necrosis factor exemplified pro-inflammatory cytokines in relative increased transcriptional activity. Interestingly, among 10 TLRs evaluated in the pathway, the mRNA expression of TLR type 4 (TLR4) was found to be increased by 1.18 fold in *C. rectus*-cells (1.18 fold upregulation), however this trend showed not to be statistically significant ($P=0.283$, T-test). All other TLRs receptors showed a non-significant down-regulation trend (Table 4.1).

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 tested the effect on TLR4 neutralizing antibodies pre-treatment in our *in vitro* system and then evaluated the production of the pro-

Table 4-1 Human Toll-Like Receptor Signaling Pathway in *C. rectus*-infected trophoblastic BeWo cells. Upregulated genes are highlighted in red.

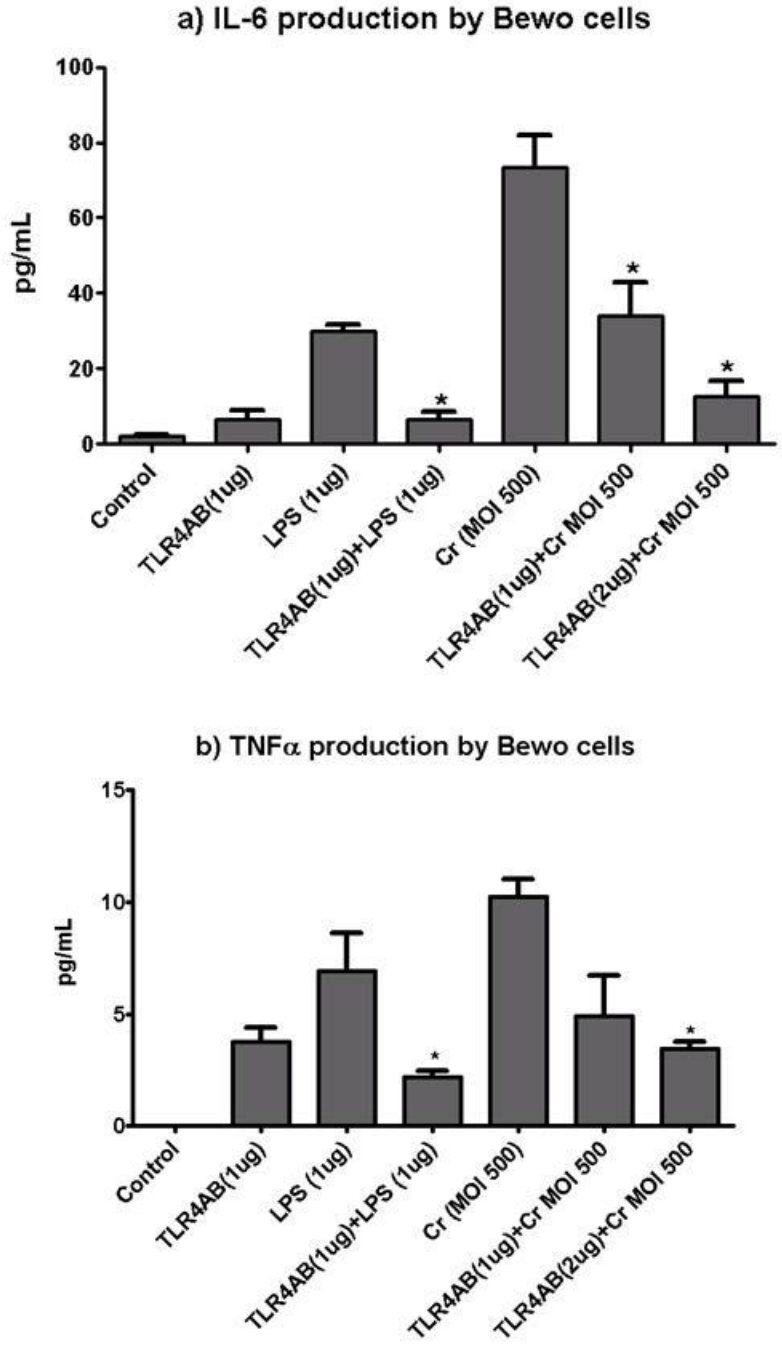
Symbol	Description	Fold Difference	T-TEST	Fold Up- or Down-Regulation
		Test Sample /Control Sample	p value	Test Sample /Control Sample
BTK	Bruton agammaglobulinemia tyrosine kinase	0.41	0.177	-2.44
CASP8	Caspase 8, apoptosis-related cysteine peptidase	1.95	0.022	1.95
CCL2	Chemokine (C-C motif) ligand 2	0.24	0.195	-4.25
CD14	CD14 molecule	0.27	0.168	-3.68
CD80	CD80 molecule	0.56	0.254	-1.78
CD86	CD86 molecule	0.32	0.167	-3.14
CHUK	Conserved helix-loop-helix ubiquitous kinase	0.68	0.274	-1.48
CLEC4E	C-type lectin domain family 4, member E	0.28	0.164	-3.57
CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	1.06	0.694	1.06
CSF3	Colony stimulating factor 3 (granulocyte)	0.32	0.167	-3.14
CXCL10	Chemokine (C-X-C motif) ligand 10	0.36	0.213	-2.76
EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2	0.40	0.035	-2.49
ELK1	ELK1, member of ETS oncogene family	0.64	0.336	-1.57
FADD	Fas (TNFRSF6)-associated via death domain	0.66	0.261	-1.52
FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog	0.55	0.083	-1.80
HMGB1	High-mobility group box 1	0.71	0.253	-1.40
HRAS	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	0.50	0.108	-1.99
HSPA1A	Heat shock 70kDa protein 1A	0.34	0.280	-2.93
HSPD1	Heat shock 60kDa protein 1 (chaperonin)	0.57	0.132	-1.76
IFNA1	Interferon, alpha 1	0.38	0.185	-2.62
IFNB1	Interferon, beta 1, fibroblast	0.41	0.177	-2.44
IFNG	Interferon, gamma	0.20	0.228	-5.09
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	0.46	0.066	-2.17
IL10	Interleukin 10	0.38	0.179	-2.61
IL12A	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	0.51	0.301	-1.97
IL1A	Interleukin 1, alpha	0.34	0.292	-2.91
IL1B	Interleukin 1, beta	0.81	0.518	-1.24
IL2	Interleukin 2	3.38	0.100	3.38
IL6	Interleukin 6 (interferon, beta 2)	2.26	0.215	2.26
IL8	Interleukin 8	0.32	0.240	-3.16
IRAK1	Interleukin-1 receptor-associated kinase 1	0.37	0.132	-2.68
IRAK2	Interleukin-1 receptor-associated kinase 2	0.21	0.129	-4.65
IRF1	Interferon regulatory factor 1	0.49	0.167	-2.05
IRF3	Interferon regulatory factor 3	0.70	0.105	-1.42
JUN	Jun oncogene	0.39	0.125	-2.60
LTA	Lymphotoxin alpha (TNF superfamily, member 1)	0.26	0.176	-3.87
CD180	CD180 molecule	0.26	0.176	-3.87
LY86	Lymphocyte antigen 86	0.52	0.268	-1.91
LY96	Lymphocyte antigen 96	0.29	0.161	-3.46
MAP2K3	Mitogen-activated protein kinase kinase 3	0.85	0.810	-1.18
MAP2K4	Mitogen-activated protein kinase kinase 4	0.82	0.437	-1.22
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	0.43	0.137	-2.31
MAP3K7	Mitogen-activated protein kinase kinase kinase 7	0.80	0.187	-1.25
MAP3K7IP1	Mitogen-activated protein kinase kinase kinase 7 interacting protein 1	0.71	0.321	-1.40
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4	0.51	0.026	-1.97
MAPK8	Mitogen-activated protein kinase 8	0.39	0.117	-2.60
MAPK8IP3	Mitogen-activated protein kinase 8 interacting protein 3	0.36	0.047	-2.80
MYD88	Myeloid differentiation primary response gene (88)	0.37	0.016	-2.71
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	0.66	0.245	-1.51
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	0.70	0.279	-1.42
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	0.62	0.288	-1.61
NFKBIL1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1	0.62	0.244	-1.60
NFRKB	Nuclear factor related to kappaB binding protein	0.50	0.054	-1.98
NR2C2	Nuclear receptor subfamily 2, group C, member 2	0.67	0.113	-1.48
PELI1	Pellino homolog 1 (Drosophila)	0.62	0.138	-1.60
PPARA	Peroxisome proliferator-activated receptor alpha	0.40	0.066	-2.50
PRKRA	Protein kinase, interferon-inducible double stranded RNA dependent activator	0.75	0.157	-1.33
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	0.42	0.187	-2.39
REL	V-rel reticuloendotheliosis viral oncogene homolog (avian)	0.26	0.125	-3.85
RELA	V-rel reticuloendotheliosis viral oncogene homolog A (avian)	0.35	0.007	-2.83
RIPK2	Receptor-interacting serine-threonine kinase 2	0.74	0.346	-1.35
SARM1	Sterile alpha and TIR motif containing 1	0.56	0.133	-1.78
SIGIRR	Single immunoglobulin and toll-interleukin 1 receptor (TIR) domain	0.27	0.226	-3.68
ECSIT	ECSIT homolog (Drosophila)	0.50	0.054	-2.01
TBK1	TANK-binding kinase 1	1.05	0.956	1.05
TICAM2	Toll-like receptor adaptor molecule 2	0.58	0.102	-1.72
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein	0.45	0.024	-2.20
TLR1	Toll-like receptor 1	0.32	0.167	-3.14
TLR10	Toll-like receptor 10	0.42	0.166	-2.36
TLR2	Toll-like receptor 2	0.26	0.175	-3.84
TLR3	Toll-like receptor 3	0.51	0.284	-1.96
TLR4	Toll-like receptor 4	1.18	0.283	1.18
TLR5	Toll-like receptor 5	0.25	0.222	-3.97
TLR6	Toll-like receptor 6	0.54	0.234	-1.85
TLR7	Toll-like receptor 7	0.34	0.178	-2.95
TLR8	Toll-like receptor 8	0.32	0.167	-3.14
TLR9	Toll-like receptor 9	0.26	0.055	-3.84
TNF	Tumor necrosis factor (TNF superfamily, member 2)	1.95	0.238	1.95
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A	122.44	0.000	122.44
TOLLIP	Toll interacting protein	0.37	0.075	-2.73
TRAF6	TNF receptor-associated factor 6	0.70	0.190	-1.44
TICAM1	Toll-like receptor adaptor molecule 1	0.52	0.003	-1.94
UBE2N	Ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)	0.39	0.022	-2.54
UBE2V1	Ubiquitin-conjugating enzyme E2 variant 1	0.94	0.840	-1.07

inflammatory cytokines IL-6 and TNFα as outcome measurements by means of multiplexing assays. As depicted in Figure 4.1, there was a dose-dependent effect in the neutralizing activity of TLR4AB in reducing pro-inflammatory activity in BeWo cells. For example, for total IL-6 production in response to MOI500 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). Similarly, 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 sizes an frequency of fetal resorptions there were no statistical differences among all experimental groups ($P>0.05$, Chi-square). Differences in fetal weight are shown in Figure 4.2. 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*, their $TLR4^{lps\text{-}d\text{-}/\text{-}}$ 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).

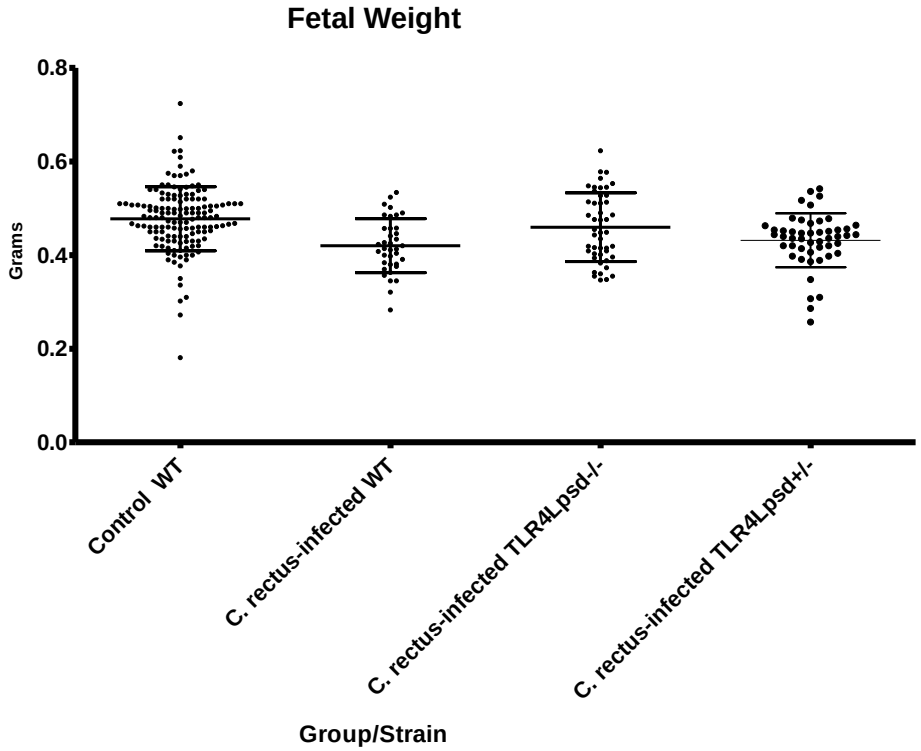
Figure 4.1 TLR4 neutralization in trophoblastic cells affects cytokine production after in vitro *C. rectus* infection.



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

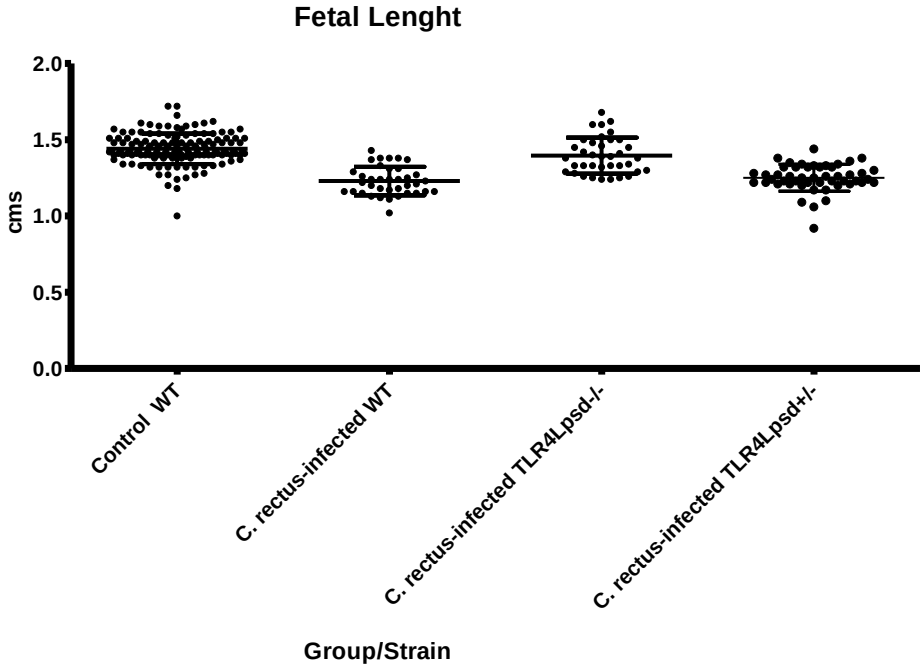
Furthermore, when TLR4$^{lps\text{-}d}$ dams were mated with a male from a different genetic background (WT) and after *C. rectus* infection, the IUGR phenotype of TLR4$^{lps\text{-}d+/-}$ heterozygous fetuses was more evident as the average fetal weight of 0.43± 0.008 was statistically different from the WT controls ($P<0.05$, Kruskal-wallis). A very similar trend was also observed for the fetal length (expressed in cms) as illustrated in Figure 4.3. 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 ($P>0.05$). Infected heterozygous TLR4$^{lps\text{-}d+/-}$ on the other hand showed again smaller fetuses (1.251±0.012) that were significantly different from fetuses from the WT controls.

Figure 4.2 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 TLR4Lpsd+/- and *C. rectus*-infected WT vs. *C. rectus-infected* TLR4Lpsd-/-. There were not significant differences (P>0.05) for Control WT vs. *C. rectus*-infected TLR4Lpsd-/-, *C. rectus*-infected WT vs. *C. rectus*-infected TLR4Lpsd+/- and *C. rectus*-infected TLR4Lpsd-/- vs. C. rectus-infected TLR4Lpsd+/-.

Figure 4.3 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 TLR4Lpsd+/-, *C. rectus*-infected WT vs. *C. rectus*-infected TLR4Lpsd-/- and for *C. rectus*-infected TLR4Lpsd-/- vs. C. rectus-infected TLR4Lpsd+/-. There were no significant differences for Control WT vs. *C. rectus-infected* TLR4Lpsd-/- and *C. rectus*-infected WT vs. *C. rectus*-infected TLR4Lpsd+/-.

Discussion

Preterm delivery affects 12.5% of live pregnancies the United States (Abrahams 2008) and is still the major cause of neonatal morbidity and mortality. Even though the etiology of pregnancy complications remains somewhat elusive, there is a strong association between infections and PTD (Goldenberg et al. 2000). Moreover, the available clinical evidence to date suggests that the way such infections could lead to prematurity involves the host immune response toward the infectious agent, leading to subsequent inflammation (Romero et al. 2007). These clinical observations 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).

For all these reasons, Toll-like receptors (TLRs) have gained a lot of attention in PTD research in the last decade. TLRs are a class of proteins that play a key role in the innate immune system. They are single, membrane-spanning, non-catalytic receptors that recognize structurally conserved molecules derived from microbes(Uematsu & Akira 2006). Once microbes breach physical barriers such as the skin, intestinal tract mucosa or the fetal maternal interphase, they are recognized by TLRs which then activate immune cell responses. In particular, TLR receptors type 4 (TLR4) have 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 & Mrinalini 2004). Furthermore,

mice deficient for TLR4 show protection against bacterial and LPS-induced PTD (Wang & 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, it has been hypothesized that Trophoblast cells, upon recognition of microbes through TLRs, may coordinate an immune response by recruiting cells of the innate immune system to the maternal-fetal interface, a key feature in PTD and Pre-eclampsia pathogenesis (Abrahams & Mor 2005). 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 2007a). Researchers have reported that trophoblastic cells can 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).

Such pro-inflammatory phenotype has also been observed in our experiments using the oral periodontal pathogen *Campylobacter rectus*. *C. rectus* is an orally-exclusive 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). Interestingly, *C. rectus* is part of the Campylobacteriacae 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 & 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 maternal inflammatory serum markers upregulation (Yeo et al. 2005), dissemination and invasion to trophoblastic cells and placental tissues and consequent placental pro-inflammatory response that ultimately result in IUGR (Arce et al. 2010).

Since we have previously reported that the expression of TLR4 at the placental level becomes upregulated in response to oral *C. rectus* exposure (Arce et al. 2009), we sought to evaluate the gene expression profile in BeWo trophoblastic cells limited to a TLR-pathway specific superarray platform (80 genes) in response to an *in vitro C. rectus* infection when compared to non-infected cells. Interestingly, a major trend toward differential down-regulation was observed in the majority of genes (90%) (Table 4.1). However, only 8 genes showed a statistically significant down-regulation trend (EIF2AK2, MAP4K4, MAPK8IP3, MYD88, RELA, TIRAP, TICAM1 and UBE2N, $P<0.05$, T test). Conversely, the minority of genes (10%) showed a trend toward differential upregulation, and only 2 of these genes were statistically significant (CASP8 and TNFRSF1A, $P<0.05$, T test). Among different members of TLRs, the only receptor that showed a non significant up-regulation trend was TLR4 (1.18 fold difference) and all others showed a non-significant down-regulation trend.

Overall, the expression of most genes in the superarray was low in both the control (non-infected) and test (*C. rectus*-infected) groups, so the quantification results tended to have greater variations. In addition to this, the number of biological replicates was limited to 3 per group which ultimately may have limited the reach of statistical significance threshold. Nonetheless, if comparing gene expression changes in terms of differential expression, these results could be interpreted as consistent with our previous findings of trophoblastic pro-inflammatory activity, in which mRNA levels for TLR4, Il-6 and TNFα have showed increased transcriptional activity (Arce et al. 2010). Similarly, other studies have been conducted to determine changes in TLR-4 expressions in trophoblasts at the placental bed of women with and without preeclampsia, finding increased TLR-4 protein expression in interstitial trophoblasts of patients with PTD (Krikun et al. 2007) or Pre-eclampsia (Kim et al. 2005).

We also have previously reported a dose-dependent response for human IL-6 and TNFα production both at the messenger (mRNA) and protein levels in human BeWo trophoblasts in response to *C. rectus in vitro* infection (Arce et al. 2010). We here aimed to determine whether the antagonism of TLR4 by means of a neutralizing TLR4 antibody (TLR4AB) would impair the observed pro-inflammatory phenotype. 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-response decrease in the production of IL-6 was observed as depicted in Figure 4.1a ($P<0.05$, Anova for both doses when compared to *Cr* MOI500). A similar trend was observed

for TNFα production (Figure 4.1b, $P<0.05$, Anova for 2ug when compared to *Cr* MOI500). These results strongly suggest 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 4.2 and 4.3, when WT dams were infected with *C. rectus*, there was a statistically significant weight dropping (12.5%) and size reduction (14.7%)($P<0.05$, Kruskall-walis). However, in the absence of functional TLR4 in homozygous fetuses, no IUGR phenotype was detected as $TLR4^{Lps\text{-}d\text{-}/\text{-}}$ fetuses were not statistically different from sham-infected WT controls for both weight and size ($P>0.05$, Dunn's). Furthermore, when fetuses carried only 1 allele with a functional TLR4 in the heterozygous $TLR4^{Lps\text{-}d+/\text{-}}$ group the IUGR phenotype was rescued 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. 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, our experimental data indicates that TLR4 is differentially upregulated in infected Trophoblastic cells over other TLRs, and when TLR4 is neutralized 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 less 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). 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.

5. Final thoughts and future directions.

We believe that the experimental results presented in this dissertation highlight the potential role of the oral bacterium *Campylobacter rectus* in eliciting pro-inflammatory responses in human placental tissues, supporting the biological plausibility of periodontal disease associations to preterm delivery and low birth weight pathogenesis. Nevertheless, data must be carefully interpreted and the study limitations must be acknowledged as some questions still remain unanswered.

We first evaluated the IUGR phenotype in response to oral infection with periodontal pathogens in Balb/C mice. When two of these pathogens (*C. rectus* and *P. gingivalis*) were applied during the oral infection period, we actually observed a significant impairment in murine fecundity; however we did not examine the biological mehanisims leading to this phenotype. While there is no known human evidence linking impaired fecundity to periodontal diseases in humans, it is interesting to speculate that the deletirous effects of oral bacteria could take place in very early phases of pregnancy, specially during trophoblast-mediated blastocyst implantation processes. This would explain in part why recent clinical trials evaluating the effects of periodontal treatment have failed to prove a significant reduction in preterm delivery cases, as periodontal treatment during the second-third trimester may be late in protecting a susceptible mother. This mechanism warrants further investigation.

When we evaluated the *in vitro* invasion mechanisms of *C. rectus* on trophoblastic cells, we were surprised to see the unresponsiveness of murine trophoblasts in the model. While we used cells of a different genetic background (SM9-1 from Swiss-Webster) than cells on a Balb/C background, the fact that murine trophoblasts did not respond to *C. rectus* exposure really limits our interpretations in the murine IUGR phenotype, as bacteria may be using different pathogenic mechanisms that we do not know about. Future research should evaluate trophoblastic cell explants from Balb/C placentas to validate or disprove these theories.

Finally, the fact that we found Toll-like receptor type 4 to be important in mediating the IUGR phenotype in mice, this model also raises many questions related to *C. rectus* recognition, invasion and clearance mechanisms during IUGR onset. For example, we only evaluated TLR4 as a proof-of-principle model but our results may be biased and they may miss other important TLRs role in the recognition process. Likewise, we could not explain if TLR4 was important in mediating trophoblastic cell invasion that would explain the observed pro-inflammatory phenotype. After pro-inflammatory cytokine upregulation, we still ignore the local and systemic effects of cytokinemia in the pathogenesis of IUGR.

Nonetheless, the experimental results here presented also demonstrated the importance of TLRs in mediating proinflammatory phenotype both *in vitro* and *in vivo* infection models in response to *C. rectus* infection. Taken together, we believe

that these results elucidate in part the maternal/fetal biological mechanisms leading to PTD in humans when exposed to oral bacteria, bringing new insights, theories and health policies in the upcoming future.

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