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Differential expression of toll-like receptors in the human placenta across early gestation

Jeffrey Pudney [b], Xianbao He [a], Zahrah Masheeb [b], David W. Kindelberger [c], Wendy Kuohung [b, **, 1], Robin R. Ingalls [a, *, 1]

[a] Section of Infectious Disease, Department of Medicine, Boston Medical Center, Boston University School of Medicine, Boston, MA, USA
[b] Division of Reproductive Immunology, Department of Obstetrics and Gynecology, Boston Medical Center, Boston University School of Medicine, Boston, MA, USA
[c] Department of Pathology and Laboratory Medicine, Boston Medical Center, Boston University School of Medicine, Boston, MA, USA

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ABSTRACT

Toll-like receptors (TLRs) are an essential component of the innate immune system. While a number of studies have described TLR expression in the female reproductive tract, few have examined the temporal expression of TLRs within the human placenta. We hypothesized that the pattern of TLR expression in the placenta changes throughout the first and second trimester, coincident with physiological changes in placental function and the demands of innate immunity. We collected first and second trimester placental tissue and conducted quantitative PCR analysis for TLRs 1–10, followed by immunohistochemistry to define the cell specific expression pattern of a subset of these receptors. Except for the very earliest time points, RNA expression for TLRs 1–10 was stable out to 20 weeks gestation. However, the pattern of protein expression evolved over time. Early first trimester placenta demonstrated a strong, uniform pattern predominantly in the inner villous cytotrophoblast layer. As the placenta matured through the second trimester, both the villous cytotrophoblasts and the pattern of TLR expression within them became disorganized and patchy, with putative Hofbauer cells now identifiable in the tissue also staining positive. We conclude from this data that placental TLR expression changes over the course of gestation, with a tight barrier of TLRs forming a wall of defense along the cytotrophoblast layer in the early first trimester that breaks down as pregnancy progresses. These data are relevant to understanding placental immunity against pathogen exposure throughout pregnancy and may aid in our understanding of the vulnerable period for fetal exposure to pathogens.

1. Introduction

The mammalian placenta is a highly specialized organ composed of both maternal and fetal cells that performs vital functions for the developing fetus during gestation. In addition to transfer of nutrients, gas exchange, elimination of waste, and hormone production, the placenta is an important site of immune defense for the fetus against maternal rejection as well as exogenous microbial insults. During pregnancy, the maternal immune system adapts to permit tolerance of the fetal allograft while maintaining defenses against harmful pathogens [reviewed in Refs. [1,2]]. Any disturbance in this tightly regulated balance may lead to a breach in immune defenses, intrauterine infection by bacteria or viruses, and obstetrical complications such as miscarriage, preterm labor, intrauterine growth restriction, and pre-eclampsia. Elucidating the etiology of intrapartum infection requires identifying not only the infectious agent but also the route of entry into the amniotic cavity.

In contrast to the formerly held belief that the amniotic tissues are sterile, a number of studies over the past two years suggest the placenta harbors a unique, low-level microbiome that may be linked to birth outcome [3–7]. Surprisingly, these studies demonstrate that the taxonomic profile of the gravid vaginal microbiome was quite dissimilar to that of the placental microbiome, favoring hematogenous dissemination over intrauterine ascension as the more common route of entry of infectious agents. It is therefore

* Corresponding author. EBRC Room 610, 650 Albany St., Boston, MA 02118, USA.
** Corresponding author. 85 East Concord St., 6th floor, Boston, MA 02118, USA.
E-mail addresses: wkuohung@bu.edu (W. Kuohung), ringalls@bu.edu (R.R. Ingalls).
[1] Contributed equally to this work.

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likely that immune defenses contained within the placenta, the barrier between maternal and fetal circulation, are critical in preventing the transmission of both commensals and pathogens.

Toll-like receptors (TLRs) are a fundamental component of the innate immune system [8] and constitute an important host defense in the placenta against intrapartum infection. This family of transmembrane receptors plays a pivotal role in the recognition of microbial ligands by the innate immune system [9,10]. Toll, initially identified as a receptor involved in embryonic development in *Drosophila* [11], was found to regulate important antimicrobial responses against fungi in the adult fly [12], an observation that set off an explosion of research to identify similar receptors in humans and mice. At least ten mammalian orthologues of Toll have been identified, and most have been implicated in cellular responses to microbial pathogens [reviewed in Refs. [13–17]. For example, TLR4 and the secreted protein myeloid differentiation factor 2 (MD-2; also known as lymphocyte antigen 96 or Ly96) are required for recognition of lipopolysaccharide (LPS); TLR2, paired with TLR1 or TLR6, recognizes bacterial lipoproteins and lipoteichoic acid; TLR5 recognizes bacterial flagellin; TLR9 recognizes CpG enriched double-stranded DNA; TLR7 and TLR8 recognize single-stranded RNA (ssRNA); and TLR3 recognizes double stranded RNA (dsRNA) [reviewed [14]]. The TLRs can be sub-divided into surface expressed TLRs (TLR1, -2, -4 and -5) and endosomal expressed TLRs (TLR3, -7, -8, and -9). Upon recognition of pathogen-associated ligands, the TLRs dimerize, initiating a signaling cascade that leads to the secretion of proinflammatory cytokines and antimicrobial peptides, induction of interferon stimulated genes (primarily from the endosomal TLRs and TLR4), as well as activation of the adaptive immune response.

In contrast to the abundant data on TLR function, the temporal expression of toll-like receptors (TLRs) throughout pregnancy has not been as well studied. It is known that TLRs 1–10 are expressed in term human placenta, and that TLR2 and TLR5 transcripts appear to rise in association with labor [18]. A recent survey of expression and function of TLRs in first trimester cytotrophoblasts suggests that TLRs are broadly expressed at 6–12 weeks gestation [19], although a comparison to second and third trimester placental tissue was not done. Knowledge of temporal shifts in TLR expression at the maternal-fetal interface may be important clinically to establish periods of increased maternal susceptibility to transmit infections such as CMV and Zika virus or optimal windows for treatment of infections that could impact the fetus. In this report, we describe the expression of TLRs in the human placenta at the level of gene expression and protein, reviewing the relevant details of placental histology over the first and second trimester.

2. Materials and methods

2.1. Study samples

First and second trimester placentas were obtained from elective termination of pregnancies under a protocol approved by the Institutional Review Board (IRB) for the Boston University Medical Campus and in accordance with the principles expressed in the *Declaration of Helsinki*. Criteria for exclusion of placental samples included known current maternal viral or pelvic infection, preexisting fetal demise, and known abnormal fetal karyotype. Termination of pregnancy was performed by suction dilation and evacuation. Samples were divided according to trimester of collection as follows: first trimester, up to 12 weeks; and second trimester, 12–24 weeks gestation. Gestational age was dated according to the subject's last reported menstrual period and/or ultrasound dating as well as foot measurement when possible. At least 2 placental specimens representing each week of gestation were obtained during the first trimester of pregnancy from 6 to 12 weeks and during the second trimester from 13 to 22 weeks. More specific numbers of samples per analysis are provided below. Placental tissue utilized for RNA analysis was stored in RNAlater (Qiagen) for later processing for RNA extraction. The remaining samples for histology were fixed in 10% unbuffered methanol-free formaldehyde and processed for embedding in wax; 5 μm thick sections were cut from each sample and placed on glass slides for histological analysis.

2.2. RNA isolation

Placental tissue was homogenized by using a Tissue Homogenizer LT (Qiagen) and TRIzol (Life Technologies). After pelleting tissue debris, tissue homogenates were transferred to a fresh tube and chloroform was added. After precipitation of protein, the RNA-rich upper aqueous phase was transferred, mixed with an equal volume of 70% ethanol, and then loaded onto RNeasy Mini Kit columns (Qiagen). All RNA was treated with RNase-free DNase (Qiagen) prior to cDNA synthesis.

2.3. Quantitative reverse transcriptase PCR analysis

A total of 11 samples were available for PCR analysis (first trimester, n = 6 samples; second trimester, n = 5 samples). Quantitative reverse transcription PCR analysis (qRT-PCR) for TLR expression was performed with the TaqMan Expression Assay system (Life Technologies), which utilizes FAM-MGB probes for each target. Primer IDs for the target genes [TLRs 1–10, MD-2, GAPDH, and N-myc downstream-regulated gene 1 (NDRG1)] are shown in Table 1 cDNA preparation was carried out using High Capacity RNA-to-cDNA kit (Life Technologies). The final 20 μl PCR reaction mixture consisted of 10 μl of 2x TaqMan Gene Expression Master mix, 1 μl of cDNA, 8 μl of RNase-free water, and 1 μl of TaqMan Gene Expression Assay mixture containing the target primer. Reactions were performed in a 96-well plate and the housekeeping gene GAPDH was used as internal control. Real-Time PCR was run on an Applied Biosystems StepOnePlus™ Real-Time PCR System using a standard program (50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15s and 60 °C for 1 min).

PCR data was analyzed using StepOne Software v2.3, and the comparative Ct method to calculate relative quantitation. The Ct from the target genes of each sample was normalized to the Ct of the reference gene GAPDH, to generate the sample delta Ct (ΔCt sample = Ct reference gene GAPDH − Ct target). To compare the gene expression levels between samples of different gestational age, the target genes were normalized to sample 1, which we defined as the earliest gestational age (sample ΔΔCt = sample ΔCt - sample 1ΔCt). To compare the different TLR gene expression levels,

Table 1
RT-qPCR primer ID, TaqMan®Gene Expression Assay.

Target	Assay ID
TLR1	Hs00413978_m1
TLR2	Hs01014511_m1
TLR3	Hs00152933_m1
TLR4	Hs00152939_m1
TLR5	Hs01019558_m1
TLR6	Hs01039989_s1
TLR7	Hs00152971_m1
TLR8	Hs00152972_m1
TLR9	Hs00152973_m1
TLR10	Hs01675179_m1
NDRG1	Hs00608387_m1
GAPDH	Hs99999905_m1