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Increased expression of toll-like receptors 2 and 9 is associated with reduced DNA methylation in spontaneous preterm labor

Scott W. Walsh, Ph.D.[a,b], **Anuja A. Chumble, M.S.**[d], **Sonya L. Washington, M.S.**[a], **Kellie J. Archer, Ph.D.**[c], **Sinem E. Sahingur, D.D.S., M.S., Ph.D.**[d], and **Jerome F. Strauss III, Ph.D., M.D.**[a]

[a]Department of Obstetrics and Gynecology, Virginia Commonwealth University, Richmond, VA, 23298-0034, USA

[b]Department of Physiology and Biophysics, Virginia Commonwealth University, Richmond, VA, 23298-0034, USA

[c]Department of Biostatistics, School of Medicine, Virginia Commonwealth University, Richmond, VA, 23298-0034, USA

[d]Department of Periodontics, School of Dentistry, Virginia Commonwealth University, Richmond, VA, 23298-0034, USA

Abstract

The cause of spontaneous preterm labor (sPTL) is not known, but it could be due to epigenetic alterations that increase the sensitivity of decidual tissue to inflammatory stimuli. We collected decidual tissue from women at term not in labor (TNL), women at term in labor (TL), and women with sPTL. Illumina Infinium HumanMethylation450 BeadChip analysis revealed significantly reduced DNA methylation for TLR-2 and TLR-9 in sPTL as compared to TL. Immunohistochemical staining documented significantly increased expression of TLR-2 and TLR-9 in decidual tissue of women with sPTL as compared to TL or TNL. TLR expression was not present in decidual cells, but localized to tissue leukocytes as revealed by staining for CD14, a macrophage antigen, and neutrophil elastase. Microarray analysis of inflammatory genes to assess innate immune response demonstrated marked increases in expression of inflammatory cytokines and chemokines in women with TL as compared to TNL. However, when sPTL was compared to TL, there was a further increase in inflammatory cytokines, and a remarkable increase in neutrophil chemokines. These results suggest that epigenetic mechanisms could play a role in increasing leukocyte infiltration, and increasing the sensitivity of decidual tissue to inflammatory stimuli that could precipitate labor prematurely.

Corresponding Author: Scott W. Walsh, Ph.D., Virginia Commonwealth University Medical Center, Department of Obstetrics and Gynecology, P.O. Box 980034, Richmond, VA 23298-0034, (804) 828-1772 - Phone, Scott.Walsh@vcuhealth.org.

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Keywords

preterm labor; DNA methylation; toll-like receptors; decidua; macrophages; neutrophils

1. Introduction

Preterm birth is the leading cause of neonatal morbidity and mortality worldwide accounting for as many as 75% of perinatal deaths (2007, Beck et al., 2010, Goldenberg et al., 2008). It is responsible for acute complications of the newborn, as well as long-term sequelae. In the United States 12.5% of births are preterm. Women experiencing preterm labor (PTL) present a number of distinct and independent clinical phenotypes, including infection, uterine over-distension (e.g., twins), hemorrhage, short cervix, stress, clinically diagnosed maternal or fetal abnormalities, but 40–45% are spontaneous PTL (sPTL) that is not explained by other factors (Myatt et al., 2012, Voltolini et al., 2013). A common factor of all births, whether term or preterm, is inflammation.

Our group recently demonstrated the presence of subclinical intrauterine infection and preserved ex vivo inflammatory status in decidual cells of women with sPTL who had no signs of clinical infection (Castro-Leyva et al., 2012). Both term and preterm intrauterine tissues can have bacteria present without overt infection and evidence is accruing that the intrauterine environment is not sterile as once thought (Jones et al., 2009, Romero et al., 2006, Steel et al., 2005, Stout et al., 2013). The presence of bacteria in both term and preterm labor suggests that the presence of bacteria alone is not sufficient to cause sPTL and raises the possibility that sPTL without clinical infection may be due to an increased sensitivity to inflammation by vaginal flora present in intrauterine tissues.

Recent evidence suggests that interaction between vaginal microbial flora and host immune response plays a significant role in sPTL (Ganu et al., 2013). The innate immune system responds to the microbiome by detecting pathogen-associated molecular patterns (PAMPs) through various germ-line encoded pattern recognition receptors. Toll-like receptors (TLRs) are the most widely studied innate sensors and play a fundamental role in pathogen recognition and activation of innate immunity (Janeway and Medzhitov, 2002, Kumar et al., 2011, Underhill and Ozinsky, 2002). Recent studies implicate TLR activated signaling pathways in fetal membranes in preterm birth associated with infection (Abrahams et al., 2013, Gillaux et al., 2011, Hoang et al., 2014). TLR-2 was markedly increased in chorioamniotic membranes in women with sPTL (Kim et al., 2004) and TLR-9 was specifically implicated in increased host response to fetal DNA in sPTL (Scharfe-Nugent et al., 2012, Phillippe, 2014).

Very little information is available concerning epigenetics and sPTL (Burris et al., 2012, Liu et al., 2013, Liu et al., 2012, Menon et al., 2012, Parets et al., 2013), although a number of factors implicate epigenetic factors (Menon et al., 2012). Stress and stress-related behaviors, such as poor diet, could play a role in influencing birth outcomes through epigenetic alterations. Maternal psychosocial stress is a main risk factors for preterm birth (Cardwell, 2013, Sanchez et al., 2013), and recent studies link psychological stress to oxidative stress (Aschbacher et al., 2013, Clerici et al., 2012, Epel, 2009, Hsieh et al., 2012, Szanton et al.,

2012, Wang et al., 2007), which can affect DNA methylation patterns (Franco et al., 2008, Hitchler and Domann, 2007, Weitzman et al., 1994). Poor diet can also affect methylation patterns (Zaina et al., 2005, Zaina and Lund, 2011), so stress and stress-related behaviors could trigger epigenetic changes.

Epigenetic alterations in TLRs could increase a women's sensitivity to inflammatory stimuli leading to sPTL. To determine if epigenetic alterations might play a role in sPTL, we determined the DNA methylation patterns in decidual tissue of women with sPTL (no clinical signs of infection) and compared the results to women with normal term labor (TL), and then correlated the expression levels of hypomethylated TLRs to leukocyte infiltration and markers of innate immune response.

2. Materials and Methods

2.1 Study Subjects

sPTL placentas were collected from pregnant women who gave birth prior to 37 weeks of gestation and had no clinical signs of infection (fever ≥100.4° F, uterine tenderness, malodorous vaginal discharge, maternal or fetal tachycardia) or preterm premature rupture of the membranes (n = 14). Although sPTL patients did not have signs of clinical infection, histologic examination of the decidua revealed evidence of acute chorioamnionitis in 6 patients. Eight sPTL patients were treated with antibiotics and 3 with betamethasone. Labor was induced in one sPTL patient, and two had Cesarean sections, one for Herpes and one for placental abruption. TL placentas were collected from women with normal pregnancies who gave vaginal birth between 37–40 weeks of gestation (n = 16). Labor was induced in 3 TL patients. Term not in labor (TNL) placentas were collected from women who underwent non-emergency Caesarian sections between 37–40 weeks of gestation for a previous Cesarean section (n = 8). Exclusion criteria were: smokers, HIV/AIDS, drug/alcohol users, pregnancies with stillborn babies, multiple fetuses, preeclampsia, lupus, congenital abnormalities, and hemorrhage. Demographic patient data are given in Table 1.

2.2 Methylation Assay

For DNA methylation analysis, decidual tissue was carefully scraped from the chorion surface of the fetal membranes. Decidual tissue from 10 women with normal pregnancy and 8 women with sPTL were processed for DNA extraction. DNA was extracted (~10 mg by weight) using QuickGene DNA tissue kit and QuickGene-Mini80 system (AutoGen, Holliston, MA). DNA was treated with RNase A (Qiagen, Valencia, CA). DNA (1μg) was bisulfite treated and run in Illumina Infinium HumanMethylation450 BeadChip (Illumina, San Diego, CA). HudsonAlpha Institute for Biotechnology (Huntsville, AL) ran the BeadChip using the protocol provided by Illumina.

2.3. Cell culture

THP-1 cells (ATCC), a monocyte/macrophage cell line, were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 50 μM 2-mercaptoethanol and 1% antibiotics and antimycotics (100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/ml amphotericin B) at 37°C in a humidified 5% CO_2 atmosphere.

Approximately 500,000 cells per ml were seeded in 5 ml of media in T-25 flasks. Cells were grown for 72 h in clean media or with 10 μM 5-Aza-2-deoxycytidine (5-Aza, Sigma-Aldrich, Saint-Louis, MO), an agent that inhibits DNA methylation when incorporated into DNA during cell division (Kendrew, 1994). Because 5-Aza degrades in solution, four separate doses were added (0 h, 24 h, 48 h and 72 h).

2.4 Immunohistochemistry

For immunohistochemistry, a rectangular section of the fetal membranes (approximately 6 cm × 4 cm) including amnion, chorion, and decidua was removed with surgical scissors. A roll with the decidua oriented towards the interior was prepared for paraffin embedding. Decidual tissue was collected for immunohistochemical staining from 7 TNL, 6 TL and 5 sPTL women. Tissues were formalin-fixed, paraffin embedded and cut into 8 μm sections with a microtome as previously described (Leik and Walsh, 2004, Shah et al., 2010, Shah and Walsh, 2007). To quench endogenous tissue peroxidase activity, slides were incubated in 3% hydrogen peroxide in methanol for 30 minutes. For antigen retrieval, slides were heat treated in 10 mM citrate buffer for 5 minutes with a pressure cooker. Tissues were immunostained with 2 μg/ml rabbit polyclonal antibody to TLR-2 (Abcam, Cambridge, MA Cat. # ab24192), 2 μg/ml rabbit polyclonal antibody to TLR-9 (Abcam, Cat. # ab37154), a 1:200 titer of rabbit polyclonal antibody to CD14 (Proteintech, Cat. #17000-1-AP), a 1:200 titer of rabbit polyclonal antibody to neutrophil elastase (Abcam, Cat. #ab21595), or rabbit IgG isotype negative control pre-diluted in phosphate buffered saline (Invitrogen, Cat #086199) using *SuperPicTure* Kit with diaminobenzidine (Invitrogen, Cat #87663), which results in a brown stain (Life Technologies, Grand Island, NY). Slides were counterstained with 1:5 dilution of Hematoxylin QS (Vector Laboratories, Burlingame, CA). The staining protocol was the same for all samples with regard to processing, incubation times and temperature.

2.5 Quantitative RT-PCR

For cell cultures, RNA extraction was performed using QuickGene RNA cultured cell kit with QuickGene Mini-80 system (AutoGen, Holliston, MA). DNase treatment was performed using Turbo DNAse kit (Ambion, Austin, TX). RNA (0.25–1 μg) was reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Quantitative RT-PCR reactions were performed with RT^2 SYBR® Green qPCR Mastermix (SABiosciences, Frederick, MD) on an Eppendorf Realplex Thermal Cycler. For each reaction, 8 ng of cDNA was used. Primers for *TLR-2*, *TLR-9* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), used as a housekeeping gene, were purchased from Integrated DNA Technologies, Coralville, IA (TLR-2 F: 5′-CCT GGC CCT CTC TAC AAA CTT-3′, R: 5′-ACT GTG TAT TCG TGT GCT GGA TA-3′; TLR-9 F: 5′-CTG CCA CAT GAC CAT CGA G-3′, R: 5′-GGA CAG GGA TAT GAG GGA TTT-3′; GAPDH F: 5′-GAT TCC ACC CAT GGC AAA TT-3′, R: 5′-AGA TGG TGA TGG GAT TTC CAT-3′). Primers for interleukin-8 (IL-8) were purchased commercially (SABiosciences, Valencia. CA). Data were normalized to *GAPDH* by the ΔΔCt method. Melting curve analysis confirmed specificity of the primers.

2.6 Inflammatory Gene Profiling

To assess innate immune response, microarray analysis of inflammatory genes and chemokines was carried out on decidual tissue samples from each group. RNA was extracted from homogenized decidual tissue using the RiboPure kit (Ambion, Austin, TX). Reverse transcription was performed with 0.2 μg RNA using the RT^2 First Strand kit (SABiosciences, Frederick, MD). Gene profiling was generated using the Human Inflammatory Cytokines and Receptors PCR Array (SABiosciences, PAHS-011). Data were analyzed with the RT^2 Profiler PCR Array Data Analysis Template v3.0 (SABiosciences).

2.7 Data Analysis

Slides were analyzed with an Olympus BH-2 microscope (Olympus, Center Valley, PA) attached to a digital camera (Olympus QColor5) using image analysis software (cellSens, Olympus). Relative differences in staining were identified by highlighting specific stained areas with yellow overlay using the “Measuring Images” tool. Data were quantified and reported as area stained (μm^2).

2.8 Statistical Analysis

Data analysis of the Infinium HumanMethylation450 BeadChip was performed using the beadarray package in R programming environment (Dunning et al., 2007). To control for multiple hypothesis testing, the p-values were subsequently used in estimating the false discovery rates (FDR) using the q-value method (Storey and Tibshirani, 2003). Methylation values (β values) range from 0 to 1 where 0 means unmethylated and 1 means fully methylated. Δβ-values indicate the difference in average methylation values between TL and sPTL women.

One-way ANOVA with Bonferroni multiple comparisons test was used to make comparisons for non-methylation data. Demographic data are presented as mean ± SD. Quantitative data are presented as mean ± SE. A probability level of $P < 0.05$ was considered significant. A statistical software application was used (Prism 6.0 for Macintosh, GraphPad Software, Inc., San Diego, CA).

3. Results

The Illumina Infinium HumanMethylation450 BeadChip analysis revealed significant decreases in DNA methylation for decidual tissue collected from women with sPTL as compared to women with TL for *TLR-2* and *TLR-9*. For *TLR-2*, there were six CpG sites and for *TLR-9* one CpG site with significantly reduced methylation (Fig. 1). Decreased DNA methylation was specific for *TLR-2* and *TLR-9*. No significant differences in methylation were detected for other TLR genes, nor for other pattern recognition receptors genes, such as C-type lectin receptors, NOD-like receptors or RIG-I-like receptors.

To examine if DNA methylation regulates gene expression of *TLR-2* and *TLR-9*, we treated THP-1 cells, a macrophage cell line, with 5-Aza to induce hypomethylation. It was necessary to use a cell line because 5-Aza induces hypomethylation by being incorporated into genomic DNA during cell division. Macrophages isolated from patients do not divide.

Treatment of THP-1 cells with 5-Aza resulted in significant increases in *TLR-2* and *TLR-9* gene expression and this was associated with a significant increase in the expression of the neutrophil chemokine, IL-8 (Fig. 2, $P<0.001$).

Reduced methylation for the *TLR-2* and *TLR-9* genes was associated with their increased expression in decidual tissue of women with sPTL as compared to women with TL or TNL. Figure 3 shows the area of staining in decidual tissue. Very little staining was present in TNL. There was more staining in TL, but the increase was not statistically significant. However, in sPTL there were marked and statistically significant increases for both TLR-2 and TLR-9 as compared to TL or TNL ($P<0.001$). Paralleling the increase in area stained for the TLRs, the area of staining for CD14 and neutrophil elastase were also significantly greater for sPTL than TL or TNL.

Figure 4 shows representative pictures of the areas of staining. TLR-2 and TLR-9 were primarily localized to leukocytes, with little or no staining in decidual cells. Increased expression of TLR-2 and TLR-9 in sPTL paralleled increases in the presence of decidual macrophages as evidenced by CD14 staining and of neutrophils as evidenced by neutrophil elastase staining. Macrophage infiltration in sPTL was for the most part uniform throughout decidual tissue, whereas the infiltration of neutrophils in sPTL was more localized.

We used microarray to determine the gene expression of inflammatory cytokines and chemokines in decidual tissue as indicators of innate immune response. Table 2 shows the fold-change in expression for selected genes. Chemokine targets listed in the Table are from Luster AD (Luster, 1998). We found marked increases in the expression of inflammatory cytokines and chemokines in women with TL as compared to TNL. However, when we compared women with sPTL to women with normal TL, there was a further increase in inflammatory cytokines and a remarkable increase in neutrophil chemokines. Results for array genes with significant increases or decreases are presented in Supplemental Tables 1 and 2 in the online supplement.

4. Discussion

The cause of sPTL is not known, but epigenetic mechanisms might provide an answer by enhancing innate inflammatory responses of TLRs. The Illumina BeadChip analysis revealed significant decreases in DNA methylation for *TLR-2* and *TLR-9. TLR-2* is especially interesting because 6 CpG sites associated with the *TLR-2* gene were significantly hypomethylated and *TLR-2* was in the top 3% of the most hypomethylated genes. TLR-2 is of interest because it is activated by the most common species of bacteria identified in women with sPTL, which are Gram-positive and mycoplasma vaginal organisms of relatively low virulence (Goldenberg et al., 2000). Therefore, increased expression of TLR-2 could increase sensitivity of intrauterine tissue to inflammation by common species of vaginal microorganisms. TLR-9 is important because microbial unmethylated CpG dinucleotides activate it, and these DNA activators would be generated as intrauterine immune cells, such as macrophages, phagocytose invading vaginal microorganisms. Activated macrophages then release chemokines to signal the infiltration of additional leukocytes. TLR-9 is also unique as it can engage both viral and bacterial nucleic acids.

Alternatively, endogenous molecules produced in response to inflammation or physiological processes recognized as damage-associated molecular patterns could activate TLR-2 and TLR-9 (Chavez-Sanchez et al., 2014, Ding et al., 2013, Gill et al., 2010, Janeesh et al., 2014, Jialal et al., 2014, Solinas and Karin, 2010, Huang et al., 2011). This would provide a sterile mechanism for engaging the innate immune system.

Reduced DNA methylation of *TLR-2* and *TLR-9* in sPTL was associated with marked and significant increases in their expression in decidual tissue. This is consistent with known epigenetic actions of DNA methylation to silence gene expression and de-methylation to increase gene expression (Seton-Rogers, 2013, Zaina et al., 2005). To evaluate if reduced DNA methylation could lead to an increase in the expression of TLR-2 and TLR-9, we used THP-1 cells, a monocyte/macrophage cell line commonly used to study macrophage function in pregnancy. Experimentally reducing DNA methylation resulted in a significant increase in gene expression for both *TLR-2* and *TLR-9*, as well as *IL-8*, a potent neutrophil chemokine that was highly expressed in decidual tissue of women with sPTL. Although terminally differentiated macrophages in the decidua may respond differently, and other factors may be involved, the significantly increased expression of TLR-2 and TLR-9 in decidua of women with sPTL is consistent with reduced methylation playing a role.

The remarkable increase in expression of TLRs in women with sPTL without clinical signs of infection or rupture of membranes is noteworthy because it suggests a premature start of the inflammatory process of labor. Our data suggest this premature start could be mediated by macrophages because decidual staining for CD14 was increased with sPTL, and our most hypomethylated gene was TLR-2, which is expressed most abundantly in monocytes/macrophages (Muzio et al., 2000). In addition, as resident sentinels, macrophages are the first alert system in decidual tissue to warn the body of danger, however if their sensitivity to microorganisms was heightened, they might initiate a premature inflammatory response precipitating sPTL.

We used a gene array to determine the expression of inflammatory cytokines and chemokines as markers of innate immune response. We found inflammatory cytokines and leukocyte chemokines were markedly increased in decidual tissue of women with TL as compared to women with TNL. When we compared TL with sPTL, we found a further increase in inflammatory cytokines and leukocyte chemokines, but what was most noteworthy was the dominance of neutrophil chemokines in sPTL and this was associated with a significant increase in the infiltration of neutrophils. It is documented that term labor is associated with inflammation and infiltration of leukocytes into myometrium and intrauterine tissues (Challis et al., 2009, Shynlova et al., 2013, Thomson et al., 1999) with a strong gene signature for neutrophils (Sharp et al., 2016). Other investigators have shown marked infiltration of leukocytes with PTL associated with infection and preterm premature rupture of the membranes (Giaglis et al., 2016, Hamilton et al., 2012). We show here that there is also marked infiltration of leukocytes with sPTL not associated with clinical symptoms of infection or rupture of the membranes. The extensive infiltration of leukocytes in sPTL could increase sensitivity of the decidua because there would be more immune cells to respond to inflammatory stimuli.

A limitation of our study is that although clinical signs of infection were not present, subclinical intrauterine infection cannot be ruled out. Six of the sPTL samples had a histological diagnosis of acute chorioamnionitis. The presence of bacteria alone, however, is not sufficient to cause sPTL because certain bacteria have been identified in both term and preterm intrauterine tissues. This was a rationale for the present study because it raises the possibility that sPTL without clinical infection may be due to epigenetic mechanisms that increase the sensitivity of decidual macrophages to the microbiota present in intrauterine tissues. Our findings of significantly reduced DNA methylation of TLR-2 and TLR-9 in sPTL being associated with a significant increase in their expression in decidual tissue are consistent with epigenetic mechanisms playing a role.

Another limitation of our study is that gestational age at sampling could have affected the TLR methylation profile since sPTL patients delivered earlier than TNL or TL patients. Gestational age is a potential confounder in studies dealing with preterm intrauterine tissues because there are no accessible gestational-age matched controls. We do not think that gestational age explains our data because decidual cells did not express TLRs, and immunostaining for TLRs correlated with leukocyte infiltration. Our methylation findings are most likely related to the infiltration of leukocytes, which express TLRs, rather than to gestational age related changes. Little is known as to how DNA methylation is affected by gestational age. One report found an overall increase in methylation from the first to the third trimester in human placental tissue (Novakovic et al., 2011), and two reports related genome-wide neonatal DNA methylation to gestational age (Bohlin et al., 2016, Knight et al., 2016). Several of the pathways identified in cord blood were related to cell aging and cellular senescence (Bohlin et al., 2016).

In conclusion, these data are consistent with epigenetic mechanisms playing a role in increasing the sensitivity of decidual tissue via increased leukocyte infiltration and expression of TLRs that might lead to a premature inflammatory response to trigger sPTL in the absence of clinical infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TLR Manuscript (Walsh) – Highlights

- DNA methylation was significantly reduced for *TLR-2* and *TLR-9* genes in decidual tissue of women with spontaneous preterm labor not associated with clinical symptoms of infection as compared to women with normal term labor.
- Reduced DNA methylation was associated with increased expression of TLR-2 and TLR-9, which was associated with a marked infiltration of macrophages and neutrophils into decidual tissue.
- Gene expression of inflammatory cytokines and chemokines was remarkably increased in women with spontaneous preterm labor.
- These data are consistent with epigenetic mechanisms playing a role in increasing the sensitivity of decidual tissue via increased leukocyte infiltration and expression of TLRs that might trigger labor prematurely in the absence of clinical infection.

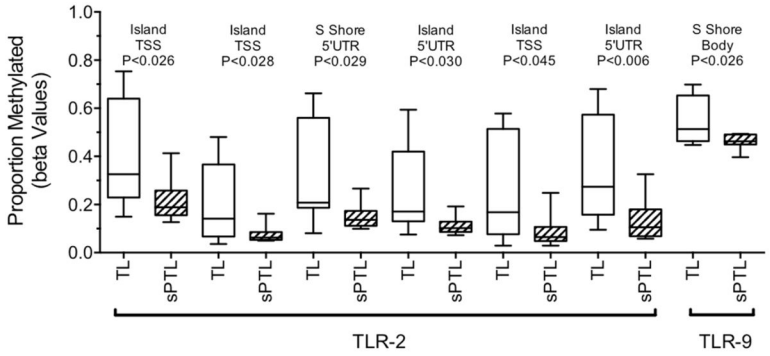

Figure 1.

Boxplots of proportion methylated (beta values) of the *TLR-2* and *TLR-9* genes in decidual tissue from women with term labor (TL, n = 10) and women with spontaneous preterm labor (sPTL, n = 8) as determined by illumina Infinium HumanMethylation450 BeadChip analysis. DNA methylation was significantly lower in sPTL than TL. The results for *TLR-2* are especially noteworthy because there were six gene related sites with significantly reduced methylation and *TLR-2* was in the top 3% of hypomethylated genes.

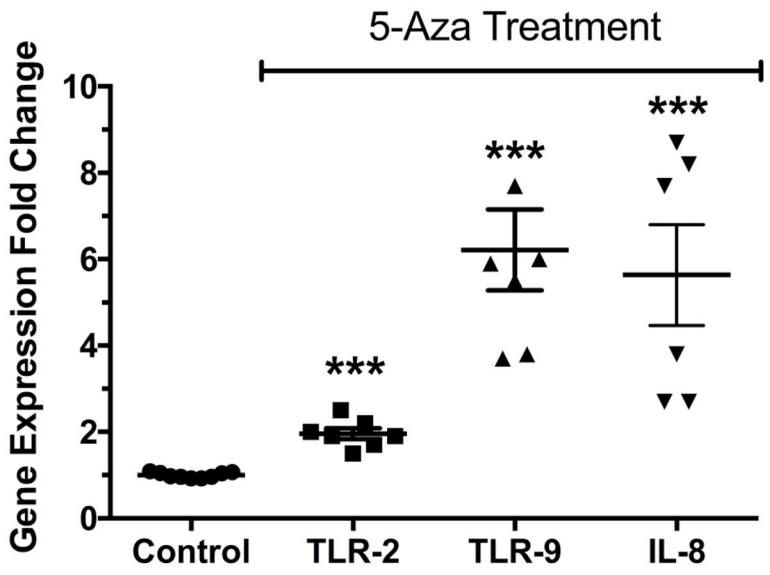

Figure 2.

TLR-2, TLR-9 and *IL-8* gene expression in THP-1 cells, a monocyte/macrophage cell line treated with 5-Aza-2-deoxycytidine, a hypomethylating agent. Treatment with 5-Aza resulted in significant increases in expression of both TLRs, as well as IL-8, suggesting *TLR-2* and *TLR-9* are regulated by DNA methylation. *** $P < 0.001$ as compared to control.

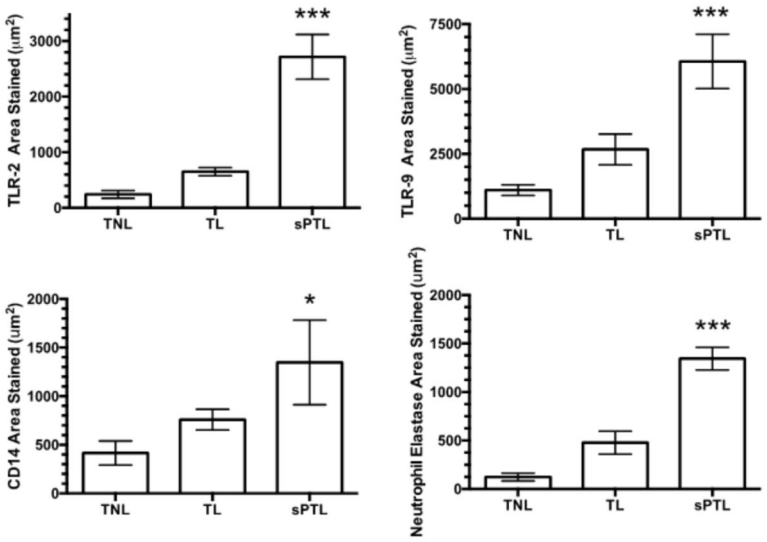

Figure 3.

Area of staining in decidual tissue for women who were at term not in labor (TNL, n = 7), women who were at term in labor (TL, n = 6), and women who delivered spontaneously preterm with no clinical signs of infection (sPTL, n = 5). The areas of staining for TLR-2, TLR-9, CD14 and neutrophil elastase were small in women with TNL or TL. However, there was a significant increase in the area stained for all markers in women with sPTL. The increase in area stained for the TLRs paralleled the increase in the area stained for macrophages as evidenced by CD14 staining and for neutrophils as evidenced by neutrophil elastase staining. Data represent mean ± SE, * $P<0.05$, *** $P < 0.001$

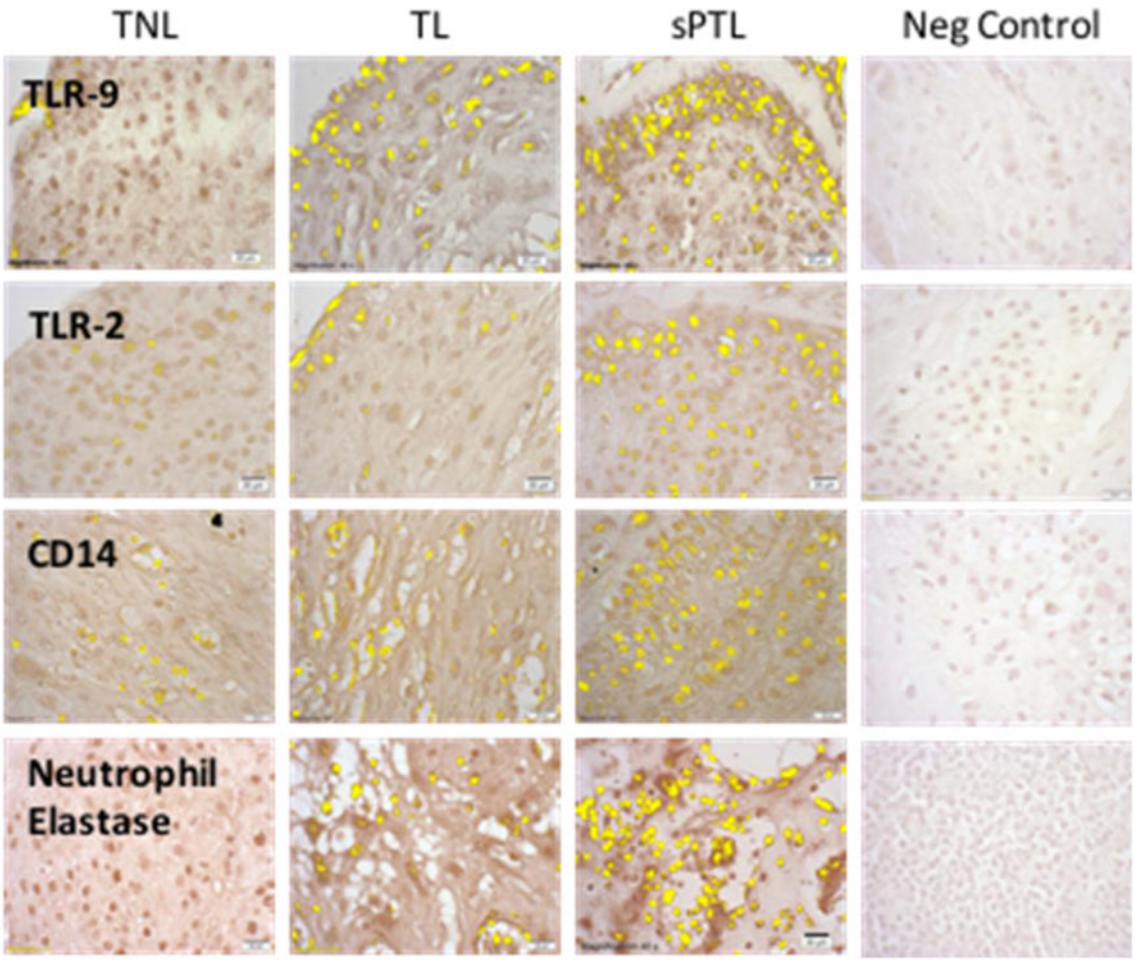

Figure 4.

Representative sections of decidual tissue from women with TNL, TL or sPTL immunostained for TLR-2, TLR-9, CD14 and neutrophil elastase. Specific antigen staining was highlighted in yellow using the Measuring Images tool in the cellSens software, which was used to determine the area of staining. There was little staining for TLR-2 or TLR-9 in women with TNL or TL and this correlated with little staining for the macrophage and neutrophils markers, CD14 and neutrophil elastase, respectively. However, in women with sPTL there was extensive infiltration of macrophages and neutrophils and significant increases in the expression of TLR-2 and TLR-9. TLR staining was primarily localized to leukocytes, rather than decidual cells. All pictures and analyses were done with a 40X lens.

Table 1

Clinical Characteristics of Patient Groups

Variable	TNL n = 8	TL n = 16	sPTL n = 14
Maternal age (y)	29.9±6.9	23.9±6.9	26.3±5.3
Pre-pregnancy BMI (kg/m^2)	28.6±8.9	25.6±5.2	23.7±8.5
BMI at sample collection (kg/m^2)	33.3±7.5	30.5±4.9	27.5±7.0
Race			
White	2	2	3
Black	4	11	10
Asian	0	0	1
Hispanic	0	1	0
Other	2	2	0
Primiparous	0	9	3
Multiparous	8	7	11
Gestational age (wk)	39.0±0.0	39.1±1.1	33.1±2.5****
Infant birth weight (g)	3230±343	3168±386	2268±510****
Delivery Method			
Vaginal	0	13	12
C-section	8	3	2

Values are mean ± SD,

**** $p < 0.0001$ compared to TL or TNL

Table 2

Fold Change in Selected Inflammatory and Chemokine Genes

TNL vs. TL			TL vs. sPTL (no clinical infection)		
Gene	**Target**	**Fold Increase**	**Gene**	**Target**	**Fold Increase**
CCL20	Dendritic cells	134	*CXCL5*	Neutrophils	111
IL-8	Neutrophils	20	*CXCL6*	Neutrophils	42
CCL2	Monocytes	17	*CXCL3*	Neutrophils	33
CCL7	Activated T cells	9	*CCL20*	Dendritic cells	25
CCL8	NK cells	7	*IL-8*	Neutrophils	9
	Dendritic cells		*CXCL1*	Neutrophils	7
IL-17C	Inflammation	7	*IL-17C*	Inflammation	5
TNF	Inflammation	7	*TNF*	Inflammation	4
IL1B	Inflammation	6	*IL1B*	Inflammation	3

TNL, term not in labor; TL, term labor; sPTL, spontaneous preterm labor.

The full list of up-regulated and down-regulated genes is given in Supplemental Tables in the online supplement.