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ARTICLE

TLR3 expression by maternal and fetal cells at the maternal-fetal interface in normal and preeclamptic pregnancies

Lobke M. Gierman[1,2] | Gabriela B. Silva[1,2] | Zahra Pervaiz[1] | Johanne J. Rakner[1] | Siv B. Mundal[1] | Astrid J. Thaning[1] | Ingunn Nervik[3] | Mattijs Elschot[4,5] | Seema Mathew[2,6] | Liv Cecilie V. Thomsen[6,7] | Line Bjørge[6,7] | Ann-Charlotte Iversen[1,2]

[1]Centre of Molecular Inflammation Research (CEMIR) and Department of Cancer Research and Molecular Medicine, Norwegian University of Science and Technology (NTNU), Trondheim, Norway

[2]Department of Gynecology and Obstetrics, St. Olavs Hospital, Trondheim University Hospital, Trondheim, Norway

[3]Cellular & Molecular Imaging Core Facility (CMIC), Faculty of Medicine and Health Science, NTNU, Trondheim, Norway

[4]Department of Circulation and Medical Imaging, NTNU, Trondheim, Norway

[5]Department of Radiology and Nuclear Medicine, St. Olavs Hospital, Trondheim University Hospital, Trondheim, Norway

[6]Department of Gynecology and Obstetrics, Haukeland University Hospital, Bergen, Norway

[7]Centre for Cancer Biomarkers (CCBIO), Department of Clinical Science, University of Bergen, Bergen, Norway

Correspondence
Lobke M. Gierman, CEMIR, Department of Clinical and Molecular Medicine, NTNU, Olav Kyrres gt. 10, NO-7491 Trondheim, Norway.
Email: lobke.gierman@ntnu.no

Abstract

Inflammation and oxidative stress at the maternal-fetal interface characterize the placental dysfunction that underlies the pregnancy disorder preeclampsia. Specialized fetal trophoblasts directly interact with leukocytes at both sites of the maternal-fetal interface; the uterine wall decidua; and the placenta. TLR3 has been implicated in the harmful inflammation at the maternal-fetal interface in preeclampsia, but the cellular involvement in the decidua and placenta has not been determined. This study aimed to characterize and quantify cell-specific TLR3 expression and function at the maternal-fetal interface in normal and preeclamptic pregnancies. TLR3 expression was assessed by immunohistochemistry and quantified by a novel image-based and cell-specific quantitation method. TLR3 was expressed at the maternal-fetal interface by all decidual and placental trophoblast types and by maternal and fetal leukocytes. Placental, but not decidual, TLR3 expression was significantly higher in preeclampsia compared to normal pregnancies. This increase was attributed to placental intravillous tissue and associated with both moderate and severe placental dysfunction. TLR3 pathway functionality in the decidua and placenta was confirmed by TLR3 ligand-induced cytokine response, but the TLR3 expression levels did not correlate between the two sites. In conclusion, functional TLR3 was broadly expressed by maternal and fetal cells at both sites of the maternal-fetal interface and the placental intravillous expression was increased in preeclampsia. This suggests TLR3-mediated inflammatory involvement with local regulation at both sites of the maternal-fetal interface in normal and preeclamptic pregnancies.

KEYWORDS

decidua, immunohistochemistry, inflammation, leukocytes, placenta, trophoblast

1 | INTRODUCTION

For a successful pregnancy an excellent collaboration between the mother and fetus is required. Maternal and fetal cellular crosstalk occurs at the two sites of the maternal-fetal interface; the maternal uterine wall decidua and the placenta.[1] The fetal cells interacting with maternal cells at both sites are the specialized trophoblasts. In the decidua, invasive extravillous trophoblasts directly interact with tissue resident maternal leukocytes. The placental surface consists of a multinucleated syncytiotrophoblast layer and underlaying

Abbreviations: CD, Cluster of differentiation; CK7, Cytokeratin 7; FGR, Fetal growth restriction; IP-10, IFN-γ-induced protein; Poly I:C, Polyinosinic : polycytidylic acid; ROD, Region of disinterest; sFlt-1, soluble FMS-like tyrosine kinase-1.

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cytotrophoblasts that cover the fetal intravillous tissue and blood vessels. The syncytiotrophoblast layer communicates directly with maternal leukocytes in the intervillous space where maternal blood is supplied from the uterine arteries. The low-grade inflammation at the maternal-fetal interface occurring in normal pregnancy is tightly regulated by local and systemic immune regulatory mechanisms and the trophoblasts serve as sensors and regulators.[2] Imbalance in the inflammatory response may have drastic impact on the maternal-fetal crosstalk and contribute to development of inflammatory disorders such as preeclampsia. Preeclampsia occurs in 2–7% of pregnancies and is a leading cause of maternal and perinatal mortality and morbidity, resulting in significant worldwide health challenges.[3-6] The disease is diagnosed with maternal end-stage signs of de novo hypertension with proteinuria or end-organ involvement after gestational week 20. Preeclampsia most often develops in two stages; first, incomplete adaption to pregnancy in the uterine wall decidua leads to placental dysfunction characterized by inflammation, endoplasmic reticulum stress, and oxidative stress; and later, exaggerated maternal response to placental stress signals leads to maternal disease.[7]

The dysfunctional placenta in preeclampsia propagates both the local inflammation and the systemic maternal response by producing increasing amounts of danger signals from damaged placental tissue.[8-10] TLRs have been given an important role in the ongoing inflammation at the maternal-fetal interface by sensing placental stress signals and orchestrate production of inflammatory cytokines and mobilization of innate and adaptive immune responses.[11] TLR3 recognizes double-stranded RNA (dsRNA) released from necrotic cells upon tissue stress or damage or as a viral replication intermediate associated with viral infection.[12,13] Previous research has suggested a role for TLR3-mediated inflammation at the maternal-fetal interface in normal and preeclamptic pregnancies,[14,15] and we have demonstrated functional placental TLR3 in primary first trimester trophoblasts.[16] TLR3 activation in pregnant mice has been shown to induce preterm delivery and placental inflammation,[14,17,18] and was involved in induction of hypertension.[19] Increased placental TLR3 expression at term has been associated with preeclampsia in a small study ($n = 5$),[15] whereas a role of TLR3-mediated inflammation in the decidua still needs to be determined. A comprehensive analysis of cellular expression and functionality of TLR3 across the two sites of the maternal-fetal interface is missing. This study aimed to characterize the cell-specific inflammatory involvement of TLR3 at both sites of the maternal-fetal interface in normal pregnancies and investigate a role for TLR3 in preeclampsia.

2 | MATERIALS AND METHODS

2.1 | Patient samples

Women with and without preeclampsia with singleton pregnancies delivering by caesarean sections were recruited at St. Olavs and Haukeland University Hospitals in Norway between 2002 and 2012. Preeclampsia was defined by persistent hypertension (systolic/diastolic blood pressure 140/90) and proteinuria (300 mg/24 h or ≥1+ by dipstick) developing after 20 wk of gestation.[20] Women with no previous history of preeclampsia or the related disorder fetal growth restriction (FGR) were included as normal pregnant controls. After delivery, a placental tissue biopsy was taken tangentially from the central part of the fetal side of the placenta in a standardized manner and a decidual sample was obtained by vacuum suction.[21] Both tissue types were fixed in 10% formalin and paraffin embedded. Metabolomics profiling of the placental tissue was used to distinguish pregnancies with normal placental function from pregnancies with moderate and severe placental dysfunction.[22]

First trimester placental tissue was collected from women undergoing surgical elective abortion at 7–12 wk of gestation. Tissues were snap frozen and stored at −80°C until fixation and paraffin embedding. Fresh decidual and placental samples from normal pregnant controls were collected after delivery for immediate isolation of explants.

Informed written consent was obtained from all participants and the study was approved by the Norwegian Regional Committee for Research Ethics (REC 2012/1040 and REC 2009/03).

2.2 | Immunohistochemistry

A total of 3 μm sections of decidual and placental tissue were incubated overnight at 4°C with a primary antibody for TLR3 (diluted 1:20 for decidua and 1:19 for placenta, #D10F10 Cell Signalling Technology, MA, USA). Serial sections were incubated with primary antibodies for the trophoblast marker cytokeratin 7 (CK7) (1:300 for decidua and 1:800 for placenta, #M7018 Dako, Glostrup, Denmark), the endothelial cell marker cluster of differentiation 31 (CD31; 1:50, #M0823 Dako) or CD45 (1:150 for decidua and 1:300 for placenta, #M0701 Dako) for 40 min at room temperature. All slides were further incubated for 30 min with HRP-labelled polymer (no. K4007; Dako). DAB+ (1:50, #K4007, Dako) was used as chromogen with two 5 min incubations and slides were counterstained with hematoxylin. CK7 decidual slides were double stained with smooth muscle actin antibodies (1:300, #M0851 Dako) with EnVision G|2 Doublestain System Rabbit/Mouse (DAB+/Permanent Red) Kit system (#K5361, Dako). Hematoxylin-eosin-saffron and negative isotype control staining was included (1:190, TLR3; Rabbit DA1E mAb #3900, Cell Signalling Technology) (Supporting Information Fig. S1).

2.3 | Explant stimulation

Fresh placentas from normal pregnancies were processed within 1.5 h of delivery and cotyledons isolated from the central part of the placenta. Decidual tissue was carefully dissected, washed in PBS supplemented with penicillin-streptomycin, and cut in pieces of similar weight (24.2 mg ± 2.1 wet weight). Placental chorionic villi were isolated by mechanically removing the fetal membrane and decidua, washed in PBS supplemented with penicillin-streptomycin, and cut in pieces (22.4 mg ± 2.6 wet weight). All explants were cultured in Ham's F12/DMEM medium supplemented with 10% FBS and 100 mg/ml penicillin-streptomycin (all from Sigma-Aldrich, St. Louis, MO, USA)

and incubated overnight at 8% O_2, 5% CO_2 and 37°C.[23] Culture medium was replaced with 250 µl fresh medium with or without TLR3 ligand, either 2–50 µg/ml polyinosinic : polycytidylic acid (poly I:C, high molecular weight, #tlrl-pic, Invivogen, San Diego, CA, USA) or Riboxxol (dsRNA duplex, #63231-63-0, Riboxx GmbH, Radebeul, Germany). Supernatants were collected after 24 h and stored at −80°C. Cell viability was assessed by lactate dehydrogenase (LDH) cytotoxicity assay (#04744926001, Roche, Indianapolis, IN, USA). For further analysis, six technical replicates for each experimental condition were combined. IL-6, IL-8, IFN-γ-inducible protein-10 (IP-10), and soluble FMS-like tyrosine kinase-1 (sFlt-1) were measured in duplicate in supernatants by quantitative sandwich ELISA (#DY206, #DY208, #DY266, #DY321B; R&D Systems, Minneapolis, MN, USA).

2.4 | Quantitative protein expression analysis

To ensure a representative analysis, large decidual tissue section scans consisting of 4–81 bright field TIFF images for each donor (20× magnification) were obtained by the EVOS FL Auto Imaging System (Thermo Fisher Scientific, Waltham, MA, USA), with defined microscope settings. A customized ImageJ ($ImageJ_2$)[24,25] script was used to perform background correction (Image calculator: Difference (img1 = |img1 − img2|) and tile stitching (grid/collection stitching plugin).[26] Decidual TLR3 expression levels were quantified using customized MATLAB scripts (version 2017b, the MathWorks, Inc., Natick, MA, USA) developed for identification and automatic quantification of staining density and intensity. A mask of patches (1325 x 1325 µm) defining trophoblasts, maternal leukocytes, and other maternal tissue was created for each decidua sample by using the serial tissue section scans stained for trophoblasts (CK7+) and leukocytes (CD45+). Muscle cells, placental tissue, blood vessels, and glands were excluded from analyses by manually defining regions of disinterest (RODs). The created masks were used to relate TLR3 expression levels to trophoblasts and maternal leukocytes in the spatially aligned images. The "expression density" of CK7, CD45, and TLR3 was calculated as the total number of positive stained pixels divided by the total amount of tissue pixels included in the analysis, to account for varying amounts of tissue between the samples. The TLR3 "expression intensity" was calculated as the average staining intensity of all positive patches. Leukocytes and trophoblasts were automatically counted and expressed as total number divided by the total area of tissue (mm^2). A color deconvolution algorithm based on DAB specific red green blue (RGB) absorption was used for quantification of protein expression.[27]

Large placental tissue section scans consisting of 4–25 bright field TIFF images for each donor (20× magnification) were taken, background corrected and tile-stitched, like for decidua. A customized MATLAB script was used to quantify TLR3 expression levels. Stem villi, decidual tissue, and tissue with poor aberrant morphology were excluded as RODs. A binary mask was created by segmentation based on RGB color values using the color threshold app in MATLAB, to select CK7, CD45, or TLR3 positive pixels. The expression density of CK7, CD45, and TLR3 were calculated as described for decidua. The expression intensity of TLR3 was measured as the average intensity of all pixels in the binary mask.

The TLR3 expression in the placental syncytiotrophoblast layer and cytotrophoblasts (n = 6–40 per placental sample) of mature villi was analyzed in three selected bright field images (20x) obtained by an Eclipse E400 microscope and DS-Fi1 camera (Nikon, Tokyo, Japan) with defined settings. The syncytiotrophoblast layer was selected in a binary layer by manually setting a threshold for CK7+ staining using NIS-Elements BR 4.0 software. Cytotrophoblast were manually selected based on morphology. The TLR3 intensity values of the syncytiotrophoblast and cytotrophoblasts were measured as grey-level intensity values ranging from 0 (absence of color, black) to 255 (presence of all colors, white) after conversion from RGB to grayscale images and calculated as the mean of positive pixels in selected areas.

All analyses were done with pregnancy outcome blinded for the observer.

2.5 | Statistical analyses

Statistical analyses were performed in SPSS (IBM SPSS Statistics 25) and GraphPad Prism (Prism7). Clinical characteristics of study objects in the two diagnostic groups were compared by unpaired t-test or Mann-Whitney test. Cytokine measurements in explant studies were analyzed by Kruskal-Wallis with Dunn's multiple comparison post hoc test. Immunohistochemistry data were analyzed by a linear mixed model. Decidual TLR3 expression levels were compared between study groups using a linear mixed model with recruitment location, study group, and the trophoblast and leukocyte densities implemented as fixed effects. Subject combinations and intercept were included as random effects. Placental TLR3 expression levels were compared between study groups using a linear mixed model with recruitment location and study group as fixed effects. Subject combinations and intercept were included as random effects. Correlations between variables were performed by calculating Pearson's correlation coefficient. Alpha level was set to 0.05.

2.6 | Online supplemental material

Supporting Information Figure S1 demonstrates negative isotype control staining for TLR3 of decidual tissue at delivery, first trimester placental tissue, and placental tissue at delivery. Supporting Information Figures S2 and S3 demonstrate immunohistochemical staining of TLR3 expression in decidual (Fig. S2) and placental tissue (Fig. S3) at delivery from normal pregnancies and preeclamptic pregnancies with moderate or severe placental dysfunction.

3 | RESULTS

3.1 | Clinical characteristics of the study group

Of the 108 pregnancies included for immunohistochemistry (Table 1), decidual biopsies from 42 normal and 48 preeclamptic pregnancies and placental biopsies from 13 normal and 28 preeclamptic pregnancies

TABLE 1 Clinical characteristics of subjects included for immunohistochemistry ($n = 108$)

	Normal pregnancies ($n = 48$)	Preeclampsia[a] ($n = 60$)
Baseline characteristics		
Maternal age, years	31.5 (±5.3)	29.5 (±5.3)
Primiparas, *n* (%)[b]	8 (17)	36 (60)
BMI[c]	24.8 (±3.9)	26.5 (±5.2)
Characteristics at time of delivery		
Systolic blood pressure, mmHg[d,e]	118.4 (±10.0)	152.7 (±20.6)*
Diastolic blood pressure, mmHg[d,e]	72.4 (±7.7)	98.7 (±11.4)*
Severe preeclampsia, *n* (%)[f]	n.a.	46 (77)
Early onset preeclampsia <34 wk, *n* (%)	n.a.	48 (80)
Placental weight, g[g]	623 (±102)	340 (±138)*
Fetal birth weight, g	3431 (±325)	1586 (±655)*
Gestational age, weeks[e]	39 (±0.7)	32 (±3.7)*

BMI, body mass index; n.a., not applicable.
Continuous variables listed as means (±SD) assessed for differences between groups by unpaired *t*-test or Mann-Whitney test. Categorical variables listed as number (percentage in column).
[a]Six of the pregnancies were diagnosed with superimposed preeclampsia and 38 were diagnosed with fetal growth restriction (FGR).
[b]Information missing from one pregnancy
[c]Maternal BMI in first trimester. Information missing from four pregnancies.
[d]Blood pressure from last healthcare visit before delivery.
[e]Information missing from two pregnancies.
[f]Preeclampsia was sub-phenotyped as severe if diagnosed with one or more severe features.[49]
[g]Information missing from 12 pregnancies.
*$P < 0.0001$ vs. normal pregnancies.

were available. Both sites of the maternal-fetal interface were available from 7 normal and 16 preeclamptic pregnancies. Maternal blood pressure was higher and gestational age at delivery and fetal and placental weight were lower in preeclamptic pregnancies compared to normal pregnancies, as expected (Table 1).

The average gestational age of the three included first trimester placentas was 10 wk (range 7–12 wk). Only information on maternal age (range 25–39 yr) and gestational age (range 267–277 d) was available for the six fresh term placentas included for explant stimulation.

3.2 | Functional expression of TLR3 at the maternal-fetal interface of normal pregnancies

TLR3 was expressed at both sites of the maternal-fetal interface. In the decidua a heterogeneous pattern of TLR3 expression was detected with strong expression in maternal leukocytes and weaker expression in trophoblasts and decidual stromal cells (Fig. 1). Placental TLR3 was expressed in the syncytiotrophoblast layer and cytotrophoblasts in both early (Fig. 1) and late pregnancies (Fig. 2A–D). A placental TLR3 expression at delivery was further observed in leukocytes, and a weaker expression was found in endothelial cells.

The decidual cell-specific TLR3 expression intensity and density was comparable between trophoblasts, maternal leukocytes, and maternal tissue (data not shown). Cell-specific quantification of the placental TLR3 expression intensity revealed comparable and highly correlated expression levels ($R^2 = 0.9$ $P < 0.001$) between the multinucleated syncytiotrophoblast and the underlying cytotrophoblasts (Fig. 2E).

When comparing the TLR3 expression at the two aligned sites of the maternal-fetal interface in normal pregnancies, neither the TLR3 expression intensity nor density showed any correlation between decidual and placental tissue (data not shown).

Explant stimulation with the TLR3 ligand poly I:C led to significant dose-dependent decidual increase of IP-10 (Fig. 3A) and placental increase of IL-6 and IP-10 ($P < 0.05$) (Fig. 3B), whereas the production of IL-8 and sFlt-1 was not affected in either tissue (Fig. 3A, B). The synthetic TLR3 ligand Riboxxol did not induce significant increase of cytokines in decidual explants (Fig. 3C) but led to significant increase of IL-6 production in placental explants (Fig. 3D). Overall, the cytokine responses were lower in decidual than in placental explants (Fig. 3A vs. B and Fig. 3C vs. D). LDH viability assay confirmed that the stimuli had no toxic effect on cell viability (data not shown).

3.3 | Expression of TLR3 at the maternal-fetal interface in preeclampsia

The same cell types expressed TLR3 at the maternal-fetal interface in preeclampsia as in normal pregnancies (Supporting Information Figs. S2 and S3). The overall decidual TLR3 expression intensity and density did not differ between normal and preeclamptic pregnancies (Fig. 4A, B, respectively). Accordingly, this was also true for the sub-analysis of TLR3 expression intensity in maternal cells and trophoblasts (Fig. 4C, D). The overall placental TLR3 expression intensity was significantly increased in preeclamptic compared to normal pregnancies (Fig. 4E, $P < 0.01$), whereas no differences in TLR3 expression density was detected (Fig. 4F). The differences in TLR3 expression intensity could not be attributed to TLR3 expression in syncytiotrophoblast (Fig. 4G) or cytotrophoblasts (Fig. 4H). Neither TLR3 expression intensity nor density showed any correlation between decidual and placental tissue from preeclamptic pregnancies (data not shown).

3.4 | TLR3 expression at the maternal-fetal interface related to the grade of placental dysfunction and leukocyte density

To further assess the TLR3 expression intensity in preeclamptic pregnancies, the grade of placental dysfunction[22] was taken into account (Fig. 4I, J). The TLR3 expression intensity in the placenta (Fig. 4J), but not decidua (Fig. 4I), was increased in preeclamptic pregnancies with both moderate and severe placental dysfunction as compared to normal pregnancies with normal placental function.

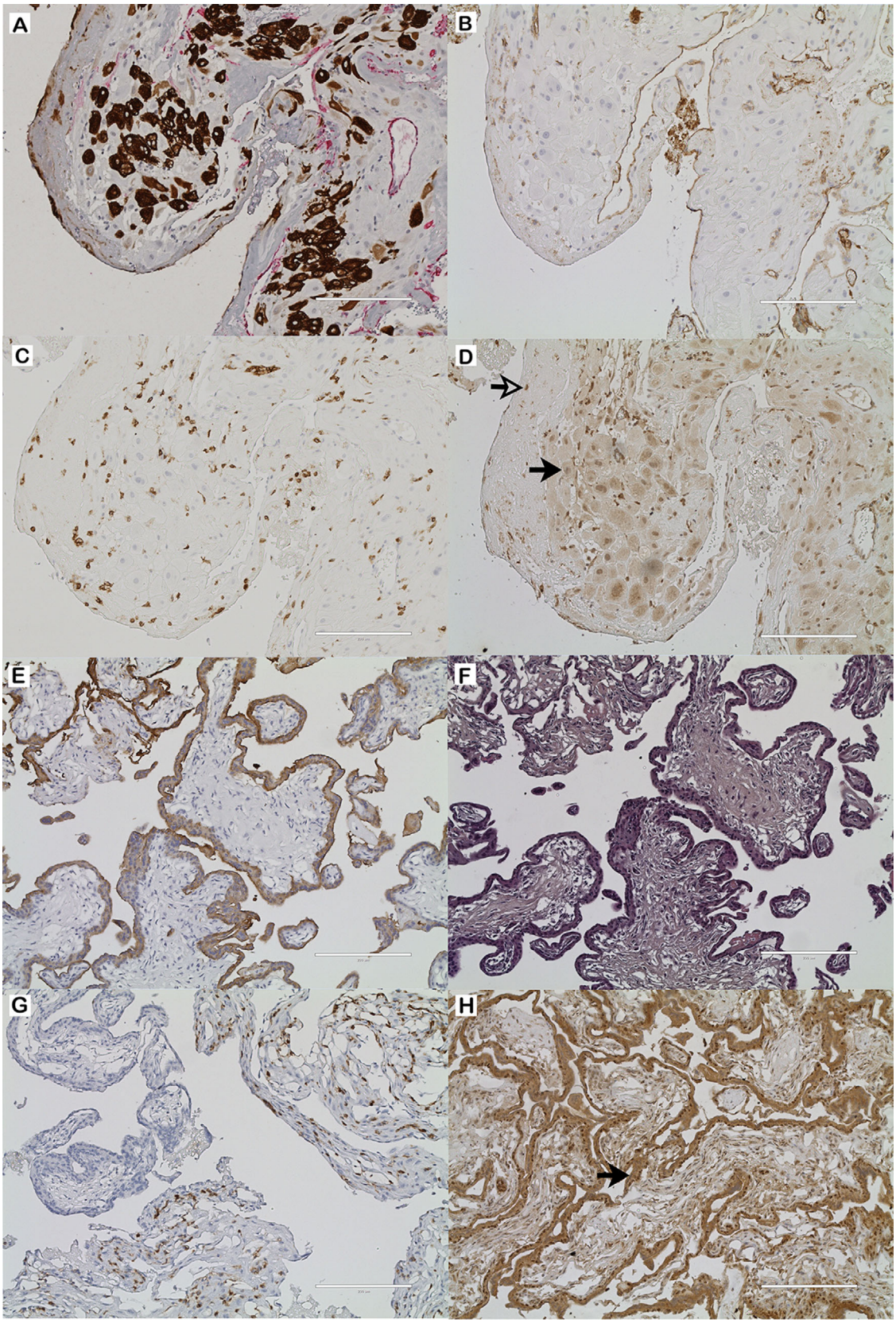

FIGURE 1 **Decidual and first trimester placental TLR3 expression.** Immunohistochemical staining of decidual tissue at delivery (**A–D**) and first trimester placental tissue (**E–H**) for trophoblasts (CK7) (**A, E**), endothelial cells (CD31) (**B, F**), and leukocytes (CD45) (**C, G**). TLR3 expression (**D, H**) in trophoblasts is indicated with a black arrow and in leukocytes with an open arrow. Decidual tissue from a normal pregnancy at gestational age 39 + 5 wk. First trimester placental tissue from gestational age 11 + 5 wk. Magnification 20×. Scale bar indicates 200 μm

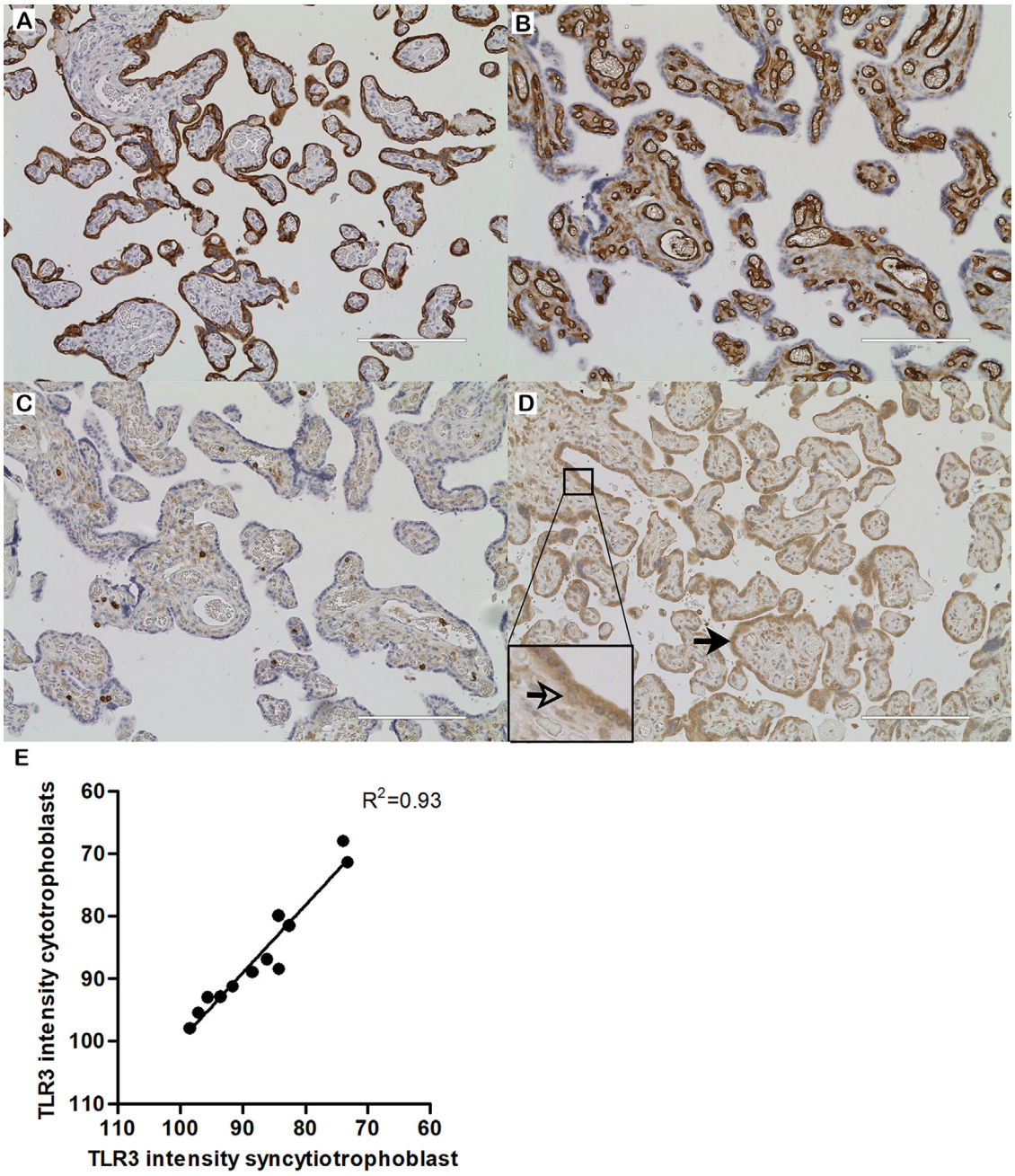

FIGURE 2 **Placental TLR3 expression at delivery.** Immunohistochemical staining of placental tissue for trophoblasts (CK7) (**A**), endothelial cells (CD31) (**B**), and leukocytes (CD45) (**C**). TLR3 expression (**D**) in the syncytiotrophoblast layer is indicated with a black arrow and a cytotrophoblast with an open arrow. Normal pregnancy at gestational age 38 + 6 wk. Magnification 20×. Scale bar indicates 200 μm. TLR3 expression intensity in the placenta at delivery of normal pregnancies ($n = 12$) was highly correlated between the syncytiotrophoblast and cytotrophoblasts ($R^2 = 0.93$, $P < 0.001$) (**E**)

The decidual and placental leukocyte density was quantified (Fig. 5) and related to TLR3 expression levels (Fig. 4). The leukocyte density was significantly higher in the decidua than in the placenta ($P < 0.001$) and showed no correlation between decidual and placental tissues. The number of decidual leukocytes did not differ between normal and preeclamptic pregnancies (Fig. 5A), whereas fewer placental leukocytes were observed in preeclamptic vs. normal pregnancies (Fig. 5B). We found no correlation between the leukocyte density (Fig. 5) and either the decidual (Fig. 4A, B) or placental (Fig. 4E, F) TLR3 expression intensity or density in normal or preeclamptic pregnancies.

4 | DISCUSSION

This study revealed functional and cell-specific TLR3 expression at the two sites of the maternal-fetal interface involving both maternal

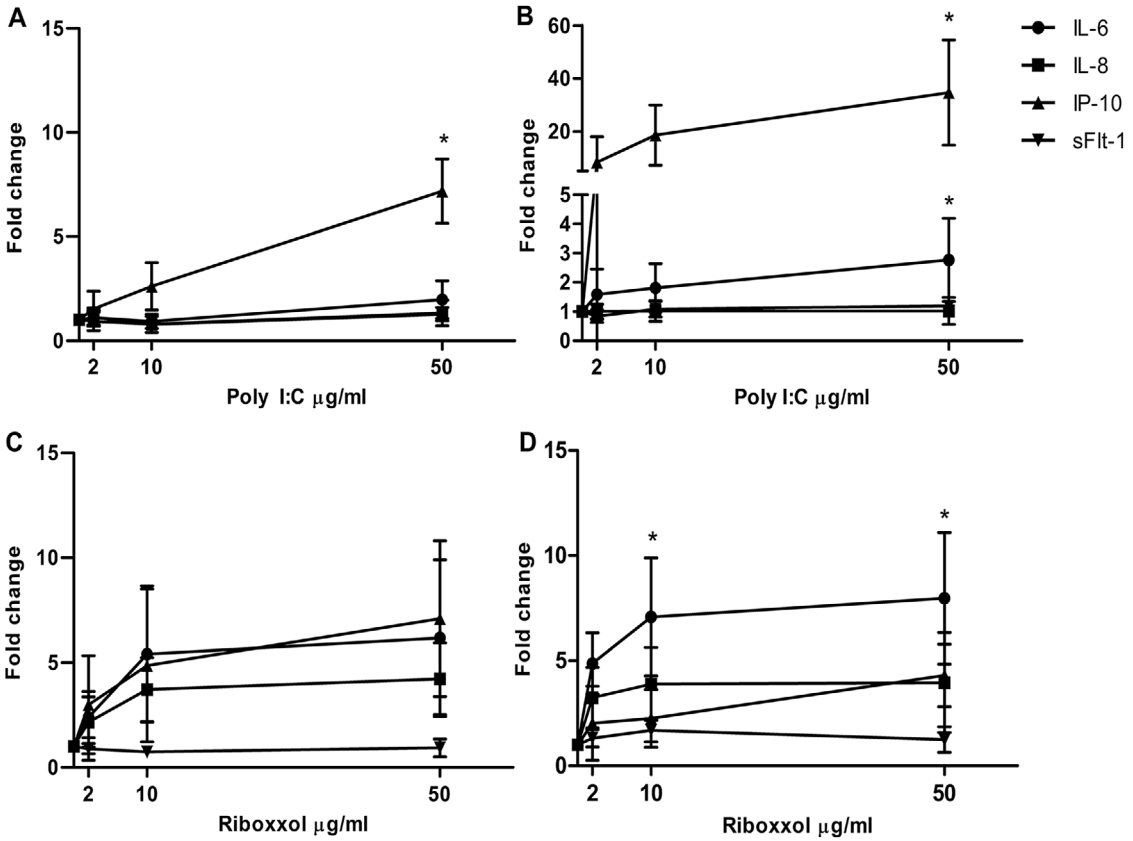

FIGURE 3 **TLR3 activated cytokine response in cultured explants.** Decidual (**A, C**) and placental (**B, D**) explants were stimulated for 24 h with 0–50 μg/ml TLR3 ligand Poly I:C (**A, B**) or Riboxxol (**C, D**). Levels of IL-6 and IL-8 ($n = 5$ for Poly I:C, $n = 3$ for Riboxxol), and IP-10 and soluble FMS-like tyrosine kinase-1 (sFlt-1; $n = 3$) were quantified in the supernatant by ELISA. Fold changes relative to unstimulated explants are presented as mean ± SD of three or five replicates. $^*P < 0.05$ vs. unstimulated

and fetal cells. TLR3 was expressed by all decidual and placental trophoblast types and by maternal and fetal leukocytes in normal and preeclamptic pregnancies, but the decidual-placental TLR3 expression levels did not correlate. Preeclampsia was associated with increased intravillous TLR3 expression. This suggests TLR3 involvement in the inflammation occurring at the maternal-fetal interface in normal pregnancies, and an added role for TLR3 in the harmful inflammation and placental dysfunction in preeclampsia, located to the intravillous placental structures and not to the direct maternal-fetal interaction sites. These findings were achieved by a novel quantitative image-based method for cell-specific assessment of protein expression in complex tissues.

The overall understanding of immune regulation at the two sites of the maternal-fetal interface is hampered by the lack of knowledge of pathologic processes in the uterine wall decidua. TLR3 gene expression in first trimester decidua[28] and functional TLR3 expression in isolated decidual macrophages and natural killer cells have been reported,[29] but this is the first quantitative characterization of cell-specific TLR3 protein expression in decidua. For placental TLR3 expression, supporting findings have been reported in early pregnancy and at delivery.[30,31] The decidua and placenta have important immunoregulatory functions throughout pregnancy and the strong TLR3 expression in maternal and fetal cells confirms an active role for TLR3 in the ongoing inflammation in normal pregnancy and the dynamic maternal and fetal cell crosstalk. This importance of TLR3 was underlined by functional pathway confirmation in both tissues. This maternal-fetal crosstalk in the decidua in late stages of pregnancy is only beginning to be characterized, but its relevance is strongly supported by the shift in decidual immune cell compositions observed in late pregnancy.[32,33] The lack of correlation for TLR3 expression between the decidua and placenta points to divergent immune regulation of TLR3 at the two sites of the maternal-fetal interface, possibly reflecting a decidual influence from maternal systemic danger signals and placental influence from placental or fetal dysregulated processes and danger signals.

The increased placental TLR3 expression in preeclampsia could not be attributed to trophoblasts in this study and this finding contrasts an existing small study.[15] Interestingly, the placental TLR3 expression pattern differed from TLR4, as we previously reported increased expression of TLR4 located to the syncytiotrophoblast in the same preeclamptic pregnancies.[8] This suggests that TLRs are differentially regulated at the maternal-fetal interface in preeclampsia and strengthens the importance of addressing distinct inflammatory pathways. The intravillous fetal structures consist of stromal cells, endothelial cells, and leukocytes. The observed increase in the cellular expression pattern of TLR3, combined with supporting literature,[15,34] suggests a role for the specialized placental macrophages, the Hofbauer

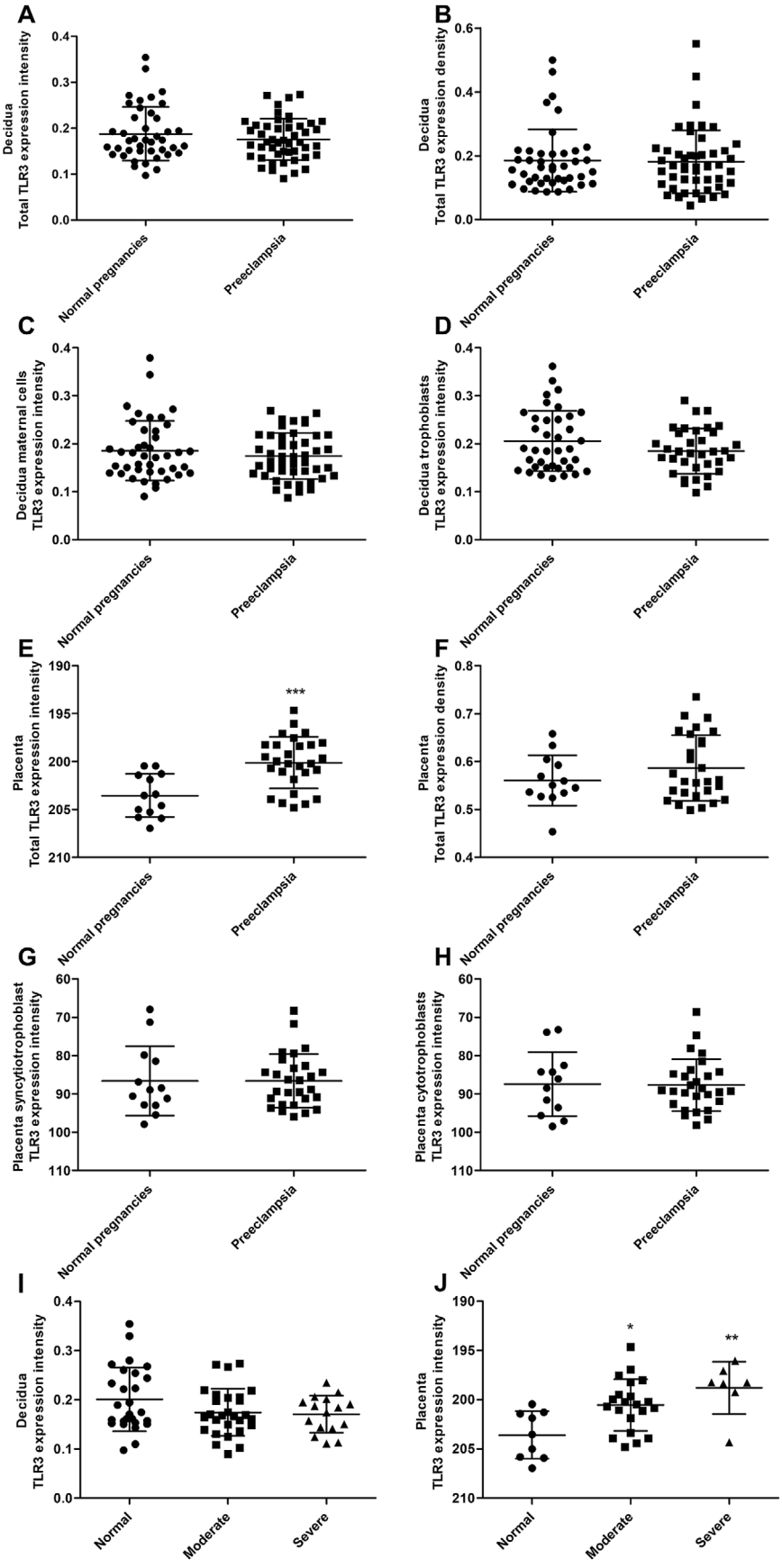

FIGURE 4 Overall and cell-specific TLR3 expression in the decidua and placenta in normal and preeclamptic pregnancies. Overall decidual (**A, B**) and placental (**E, F**) TLR3 expression intensity (**A, E**) and density (**B, F**) is shown. Cell-specific TLR3 expression intensity in maternal cells (**C**) and trophoblasts (CK7+) (**D**) is shown in decidual tissue from normal ($n = 42$) and preeclamptic ($n = 48$) pregnancies at delivery. TLR3 expression intensity in maternal cells was calculated from tissue areas without trophoblasts (CK7-) and TLR3 expression intensity in decidual trophoblasts was calculated from tissue areas with >50% trophoblast density. Placental cell-specific TLR3 expression intensity in the multinucleated syncytiotrophoblast (**G**) and cytotrophoblasts (**H**) was analyzed in normal ($n = 13$ for syncytiotrophoblast and $n = 12$ for cytotrophoblasts) and preeclamptic ($n = 28$) pregnancies at delivery. Overall TLR3 expression intensity was determined in the decidua (**I**) from normal pregnancies with normal placentas ($n = 27$), preeclamptic pregnancies with moderate placental dysfunction ($n = 28$) and preeclamptic pregnancies with severe placental dysfunction ($n = 16$); and in the placenta (**J**) from normal pregnancies with normal placentas ($n = 9$), preeclamptic pregnancies with moderate placental dysfunction ($n = 21$) and preeclamptic pregnancies with severe placental dysfunction ($n = 7$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P = 0.001$ vs. normal pregnancies

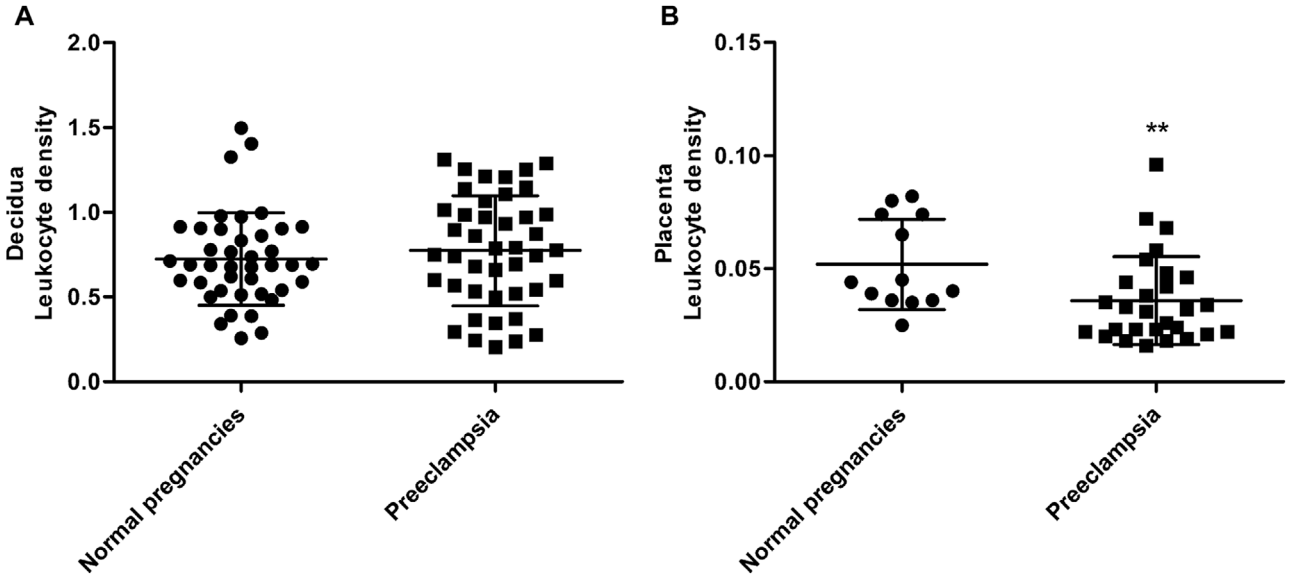

FIGURE 5 **Leukocyte density in the decidua and placenta in normal and preeclamptic pregnancies**. Leukocyte density (CD45+) in decidual (**A**) and placental (**B**) samples from normal ($n = 41$ and $n = 13$, respectively) and preeclamptic ($n = 43$ and $n = 28$, respectively) pregnancies at delivery was calculated as total positive CD45 pixels dived by the total area. $^{**}P = 0.01$ vs. normal pregnancies

cells, but this needs further characterization. The TLR3 expression in Hofbauer cells observed in early pregnancy is supported by others,[31] and suggests importance for placental immune activity throughout pregnancy. The increased intravillous TLR3 expression in preeclampsia cannot be explained by a change in leukocyte numbers, because preeclampsia was associated with reduced leukocyte density, an observation supported by others.[35] This reduction in important immune cells in preeclampsia is thought to negatively influence the placental homeostasis.[36,37] Stromal cells and endothelial cells could also be responsible for the observed increase in intravillous TLR3 expression as supported by a small study ($n = 5$) from Pineda et al.[15] Villous mesenchymal stromal cells have been shown to produce more inflammatory cytokines, chemokines, and growth factors in preeclampsia compared to normal pregnancies and this may contribute to placental dysfunction and the exacerbated inflammatory response.[38] Placental angiogenesis is impaired in preeclampsia and TLR3 activation in mice has been shown to cause endothelial dysfunction comparable to in women with preeclampsia.[14] However, the biologic significance of TLR3 expression in these cells has not yet been determined. The TLR3 expression in cells involved in the direct maternal-fetal interaction in the decidua and placenta was not influenced in preeclampsia. This suggests that the role of TLR3 in maternal-fetal communication is restricted to the physiologic inflammation of normal pregnancies and not affected by the placental dysfunction in preeclampsia.

In addition to being activated by dsRNA from viral infections,[39] TLR3 may be activated by mRNA released from necrotic cells or noncoding RNA from ultraviolet B-irradiated cells to generate a robust immune response.[13,40] The delivery and exposure of these endogenous ligands to the endosome-restricted TLR3 is still poorly understood. The natural activators of TLR3 during pregnancy could be derived from shed trophoblast debris, exosomes, and danger signals, which are released from placental tissue locally and to the maternal circulation as part of the low-grade inflammation in normal pregnancies, and this shedding is markedly increased in preeclampsia.[41] Preeclampsia is further associated with apoptosis of villous cytotrophoblasts and the potential local release of dsRNA might explain why TLR3 was increased only in intravillous cells.[42] Other sources for naturally available danger signals may potentially be the placental microbiome[43] adding potential TLR3 ligands at the maternal-fetal interface. We used high molecular weight poly I:C and Riboxxol to activate TLR3, but the activation of other cytosolic receptors such as retinoic acid inducible gene 1 and melanoma differentiation-associated protein 5[44] may possibly have contributed to the cytokine response.[45] The efficiency of TLR3 activation by different types of dsRNA may be influenced by their size as smaller dsRNA may penetrate the cells more efficiently.[46] Accordingly, the smaller Riboxxol (50 bp) induced a higher cytokine response in stimulated explants compared to the high molecular weight poly I:C (1.5–8 kb).

The heterogeneous disorder preeclampsia consists of distinct subtypes that go beyond the current diagnostic measures.[22,47] Different ongoing pathologic processes at the maternal-fetal interface might give the same clinical symptoms in the preeclamptic mother and a better classification of the disorder taking placenta dysfunction into account would lead to improved understanding, screening, and treatment of the disease. Approximate subgroups such as preeclampsia with early or late onset, and with and without FGR are often used and increasing evidence points to divergent roles for inflammatory activation in distinct phenotypes.[48] The increased placental TLR3 expression associated with preeclampsia was not affected by FGR or superimposed preeclampsia, but with both moderate and severe placental dysfunction.[22] This shows that the regulation of placental TLR3 holds true for preeclamptic pregnancies directly affected by a dysfunctional placenta and therefore possess disturbances of the physiologic processes occurring at the maternal-fetal interface.

In summary, functional TLR3 was broadly expressed by maternal and fetal cells at both sites of the maternal-fetal interface and the placental intravillous TLR3 expression was increased in preeclampsia. Our findings suggest that TLR3 is actively involved in the low-grade inflammation characterizing maternal-fetal interaction of normal pregnancies, with local regulation in the decidua and placenta. TLR3 is shown to play a role in the placental dysfunction in preeclampsia located to intravillous tissue and not to the direct maternal-fetal communication. This study underlines the importance of detailing the regulation of cell-specific pathologic processes at the maternal-fetal interface for more well-funded understanding of disease development.

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AUTHORSHIP

L.M.G. and A.-C.I. designed the study, interpreted the data, and drafted the article. G.B.S., S.M., L.C.V.T., and L.B. collected the clinical material and information. L.M.G., G.B.S., Z.P., J.J.R., S.B.M., A.J.T., I.N., M.E., and A.-C.I. developed and/or performed the quantitative immunohistochemistry analyses. L.M.G. and Z.P. performed the explant experiments. All authors critically revised the article and approved the final version.

DISCLOSURES

The authors declare no conflicts of interest.

ORCID

Lobke M. Gierman https://orcid.org/0000-0003-4328-4392

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SUPPORTING INFORMATION

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