Placental inflammation by HMGB1 activation of TLR4 at the syncytium

Short title: Placental inflammation by HMGB1 and TLR4

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Abbreviations: CK, cytokeratin; FGR, fetal growth restriction; HMGB1, high mobility group box 1; CRP, C-reactive protein; IL, interleukin; IP, interferon (IFN)-γ-inducible protein; sFlt, soluble fms-like tyrosine kinase; TLR, Toll-like receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor

Keywords

High mobility group box 1; inflammation; placenta; preeclampsia; toll-like receptors; trophoblast

Abstract

Introduction: Normal pregnancy is characterized by an elevated inflammatory state involving the placenta. The placental inflammation is further increased in preeclampsia, resulting in release of harmful danger signals to the maternal circulation. Activation of toll-like receptors (TLR)2 and TLR4 by endogenous danger signals plays a role in inflammatory diseases. Placental TLR2 and TLR4 expression has been reported, and high mobility group box 1 (HMGB1) is a likely endogenous activator of these receptors. We aimed to examine HMGB1 activation of TLR2 and TLR4 as mechanisms of placental inflammation in normal and preeclamptic pregnancies, by combined analysis of expression and function of the ligand HMGB1, the receptors TLR2 and TLR4, and the cytokine responder interleukin (IL)-8.

Methods: Protein expression was analyzed in placental tissue from normal and preeclamptic pregnancies, and cytokine responses to two distinct HMGB1 isoforms

were examined in placental explants and trophoblasts. Inflammatory and anti-angiogenic markers were measured in maternal serum.

Results: We demonstrated strong co-localized expression of HMGB1, TLR4 and IL-8 in the syncytium layer of the placenta. Syncytium TLR4 expression and maternal serum levels of IL-8 were significantly increased in preeclamptic compared to normal pregnancies. Functionality was confirmed by TLR4-dependent release of IL-8 from placental explants and trophoblasts in response to the inflammatory isoform of HMGB1.

Discussion: This demonstrates a role for the HMGB1-TLR4 pathway at the syncytium layer and suggests involvement in placental inflammation and preeclampsia**.**

Introduction

Preeclampsia is described as an excessive maternal inflammatory response to a dysfunctional placenta or to pregnancy itself [1]. Impaired remodeling of the uterine arteries that supply blood to the placenta with subsequent insufficient blood flow may contribute to oxidative and inflammatory stress [1, 2]. The damaged placenta releases increasing amounts of inflammatory cytokines, anti-angiogenic factors and other danger signals to the maternal circulation, thereby aggravating maternal systemic endothelial dysfunction resulting in clinical manifestation of preeclampsia [2, 3]. The mature placenta is covered by the syncytium, a continuous layer of fused fetal cytotrophoblasts directly exposed to maternal blood [4]. Cytotrophoblasts underlie the syncytium and are important for placental metabolism and maintenance of the syncytium [5]. The syncytium represents an important maternal-fetal interface that may be activated by inflammatory processes in the placenta and danger signals in maternal blood [6]. The harmful placental inflammation in preeclampsia is an exacerbation of normal pregnancy [1, 3]. Preeclampsia is often accompanied by fetal growth restriction (FGR) but whether these disorders share the underlying placental complications is uncertain [7]. Inflammation can be triggered by endogenous danger signals released to the extracellular environment by tissue damage such as trauma or ischemia/reperfusion injury [8]. High mobility group box 1 (HMGB1) is a nuclear transcription factor acting as a danger signal through Toll-like receptor (TLR)2 and TLR4 when released extracellularly [9, 10]. HMGB1 is a key inflammatory mediator in stroke and atherosclerosis [11] and its extracellular function depends on posttranslational redox modifications resulting in different isoforms [12]. The cytokine isoform of HMGB1 has a C23-C45 disulfide bond and initiates inflammation through TLR4 [13], resulting in

release of inflammatory cytokines like interleukin (IL)-8 and tumor necrosis factor (TNF)-α [9, 14, 15]. The HMGB1 isoform with all cysteines reduced is a chemoattractant involved in tissue regeneration [13, 16, 17].

The pathological processes necrosis [18], hypoxia [19] and oxidative stress [20] induce HMGB1 and are central to placental dysfunction in preeclampsia [1]. Maternal serum HMGB1 is elevated in preeclampsia [21-24] and the inflamed placenta is a likely source [25-28]. A role for HMGB1, TLR2 and TLR4 in placental inflammation in preeclampsia has been suggested by overall inconclusive separate expression studies in trophoblasts or placental tissue [24, 29-36]. The cell-specific distribution of these inflammatory pathways in the placenta therefore needs further characterization. We aimed to perform a comprehensive analysis of cellular distribution and functionality of HMGB1 activation of TLR2 and TLR4 to understand the contribution to placental inflammation in normal and preeclamptic pregnancies.

Methods

Ethical approval

This study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics; the Preeclampsia Study (approval no. 2012/1040) and the First and Third Trimester Study (approval no. 2009/03). Informed written consent was obtained from all participants before enrollment.

Study population

The Preeclampsia Study includes healthy and preeclamptic singleton pregnancies with or without FGR recruited during 2002-2012 at St. Olavs and Haukeland University Hospitals, Norway. Maternal venous blood was collected prior to delivery and placental tissue after delivery by cesarean section without labor. To minimize differences in gestational age, additional maternal venous blood samples were collected from healthy pregnant women at gestational age 28–32 weeks recruited in 2013-2014. Non-pregnant women were enrolled at St. Olavs Hospital in 2013 for sampling of venous blood between days 6 and 11 of the menstrual cycle.

Blood samples were left to clot for ≥ 30 min, centrifuged at 1800 x *g* for 10 min, and serum aliquots stored at -80°C. Third trimester placental biopsies were taken tangentially from the central part of the maternal side and directly fixed in 10% neutral-buffered formalin before being paraffin embedded.

Preeclampsia was defined as persistent hypertension (blood pressure ≥ 140/90 mmHg) plus proteinuria (≥ 0.3 g/24 h or reproducible ≥ 1 + by dipstick) developing after 20 weeks of gestation [37]. FGR was either diagnosed by serial ultrasound measurements or established if the neonates had a birth weight < 5th percentile according to Norwegian

reference curves [38]. Healthy normotensive pregnant women were included as normal pregnancy controls.

The First and Third Trimester Study includes placental tissue from healthy singleton pregnancies recruited at St. Olavs Hospital during 2009-2014. First trimester placental tissue from surgical elective abortions (gestational age 7–12 weeks) were snap frozen and stored at -80°C until fixation and paraffin embedding. Third trimester placentas were collected from deliveries by cesarean section for immediate isolation of chorionic villous explants. Only the phenotypic parameters maternal and gestational age were obtained for these pregnancies.

Clinical characteristics of study subjects

Twenty-three preeclamptic and 13 healthy women were included in protein expression analyses of third trimester placentas, and among the preeclamptic pregnancies, 52% were additionally complicated by FGR and 70% experienced severe preeclampsia (Table 1). Thirty-four preeclamptic women, 43 healthy pregnant women and 28 healthy non-pregnant women were included in serum analyses, and among the preeclamptic pregnancies, 59% were additionally complicated by FGR and 79% had severe preeclampsia (Table 2). The gestational age ranges were 7–12 weeks for the ten first trimester placentas, and 38–39 weeks for the five healthy third trimester placentas included for isolation of explants.

Immunohistochemical staining and quantification

Tissue sections (3 μm) were pre-treated in PT link (#PT101, Dako) using target retrieval solution (#K8005 or #K8004, Dako) at 97°C for 20 min and peroxidase blocking

solution for 5 min (#K4007, Dako). Slides were stained for TLR2 (1:500, #AF2616, R&D Systems), TLR4 (1:175, #MAB14783, R&D Systems), IL-8 (1:200, #60141-2-ig, Proteintech), HMGB1 (1:80, #MAB1690, R&D Systems), CK7 (1:800, #M7018, Dako), CD31 (1:50, #M0823, Dako) or CD45 (1:300, #M0701, Dako). For detection of TLR2, 20 min incubation (1:400, #E0466, Dako) was followed by HRP-labeled streptavidin (#K1016, Dako) for 30 min. All other slides were incubated for 30 min with HRP-labeled polymer (#K4007, Dako). DAB+ (1:50, #K4007, Dako) was used as chromogen with two 5 min incubations and slides counterstained with hematoxylin. Mouse linker (#K8021, Dako) was used for TLR4 and cell marker staining. Immunohistochemistry was performed in Autostainer Plus (#S3800, Dako). Negative isotype controls were included (Fig. 1 C, F, I, L and Fig. S1 D, F, H). Bright field images were obtained with an Eclipse E400 microscope and DS-Fi1 camera (Nikon) or the EVOS™ FL Auto Imaging System (Invitrogen). The syncytium staining intensity was quantitatively assessed by automated image analysis using the NIS-Elements BR 4.0 software (Nikon). The syncytiotrophoblast covering villous trees was delineated in a binary layer by manual adjustment. This binary layer defined the area included in automatic assessment of intensity values (the statistical mean of intensity pixel values). For HMGB1, positively stained syncytium nuclei were removed from analysis by a staining intensity threshold. The staining intensity score and protein expression level were inversely proportional. Three pictures per placental tissue section were taken of randomly selected areas with well-preserved placenta morphology, at 20x magnification. Protein expression was quantified in 13 healthy and 23 preeclamptic pregnancies, with the examiner blinded to pregnancy outcomes.

Placental explants and trophoblasts

A cotyledon was taken from the central region of fresh third trimester placentas and the fetal membrane and decidua basalis were removed. The chorionic villous tissue was washed in sterile phosphate-buffered saline supplemented penicillin-streptomycin and cut into pieces (explants) of similar weight (26.7 ± 8.0 mg). Explants were cultured in Ham's F12/DMEM medium supplemented 10% FBS and 100 mg/ml penicillin-streptomycin (Sigma-Aldrich) and incubated overnight at 37°C, 8% O_2 and 5% CO_2 [39]. The trophoblast cell line SGHPL-5, generously provided by Prof. Whitley (St. George's University of London, UK) [40] was chosen based on its functional TLR profile [41]. SGHPL-5 cells (passage 19-20) were seeded at 1 x 10^4 cells/well in Ham's F10 medium supplemented 10% FBS, 2 mM L-glutamine and 100 mg/ml penicillin-streptomycin and incubated overnight at 37°C, 20% O_2 and 5% CO_2. Fresh culture medium with or without the cytokine HMGB1 isoform (#HM-120) or the chemokine HMGB1 isoform (#HM-114) (IBL International) at 0.1–10 µg/ml (explants) or 1.25–20 µg/ml (trophoblasts) were added and supernatants collected after 24 h (explants) or 4, 24 and 72 h (trophoblasts), centrifuged and stored at -80°C. Viability was assessed by LDH cytotoxicity assay (#04744926001, Roche) (explants) or MTT analysis (trophoblasts), showing that the stimuli had no toxic effects (data not shown). Specificity was tested by adding 10 µM TLR4 inhibitor (#CLI-095, InvivoGen) [42, 43] one hour prior to HMGB1 stimulation. IL-1β, IL-6, IL-8, IL-9, IL-10, IL-12p70, interferon (IFN)-γ-inducible protein (IP)-10, TNF-α, IFN-γ and vascular endothelial growth factor A (VEGF-A) in explant (1:50) and SGHPL-5 cell supernatants (undiluted) were analyzed by a customized multiplex cytokine assay (Bio-Rad Laboratories). For TLR4-inhibition experiments, IL-8 in supernatants from third

trimester placental explants (1:1000) and SGHPL-5 cells (1:4) was measured in duplicate by sandwich ELISA (#DY208, R&D Systems).

Serum measurements

HMGB1 and soluble fms-like tyrosine kinase-1 (sFlt-1) were measured in duplicate serum samples by sandwich ELISA (#ST51011, IBL International and #DVR100B, R&D Systems, respectively). The intra- and inter-assay coefficient of variance for the HMGB1 ELISA was 0.3-6.5% and 20.8%, respectively. IL-8 was analyzed in maternal serum by cytokine assay (Human Cytokine Group I multiplex panel, # M50-0KCAF0Y, Bio-Rad Laboratories). The inflammatory marker C-reactive protein (CRP) (high sensitivity, turbidimetric assay, Modular P analyzer, Roche) was measured at St. Olavs Hospital.

Statistical analyses

Statistical analyses were performed in GraphPad Prism version 6.0 and SPSS version 21 or 23 with $P < 0.05$ considered statistically significant. The specific tests used are described in corresponding table and figure legends and includes two-sided t-tests or Mann-Whitney U-test (comparing two groups), one-way ANOVA with Tukey's post-hoc test or Kruskal-Wallis with Dunn's test (comparing three groups), or Chi-square test (categorical variables). Correlations was calculated using Pearson’s test or Spearman's rank test.

Results

Cell-specific placental expression of HMGB1 pathway components in preeclamptic and healthy pregnancies

Cell-specific placental staining outlined the multinucleated syncytium layer (CK7), with underlying distinct cytotrophoblasts (CK7), fetal endothelial cells (CD31) and leukocytes (CD45) within the villous structures (Fig. S2).

In third trimester placentas, strong expression of HMGB1 (Fig. 1A,B), TLR4 (Fig. 1G,H) and IL-8 (Fig. 1J,K) was detected particularly in the syncytium and underlying cytotrophoblasts, and both nuclear and cytoplasmic HMGB1 expression was apparent. In addition, fetal endothelial cells and villous stromal cells expressed HMGB1, TLR4 and IL-8 (Fig. 1A,B,G,H,J,K). TLR2 was expressed by leukocytes and endothelial cells, but not by the syncytium or cytotrophoblasts (Fig. 1D,E). The prominent third trimester syncytium HMGB1, TLR4 and IL-8 staining was quantified (Fig. 2). Cytoplasmic HMGB1 expression in the syncytium was higher in preeclamptic compared to normal pregnancies but did not reach statistical significance (preeclampsia 109 A.U. ± 7, preeclampsia+FGR 104 A.U. ± 8, healthy 110 A.U. ± 5, $P = 0.079$) (Fig. 2A). Syncytium TLR4 expression was significantly higher in preeclamptic compared to healthy placentas both in presence and absence of FGR (preeclampsia 94 A.U. ± 8, preeclampsia+FGR 98 A.U. ± 14, healthy 110 A.U. ± 13, $P = 0.006$) (Fig. 2B). The syncytium IL-8 expression levels did not differ between placentas from preeclamptic and healthy pregnancies (preeclampsia 146 A.U. ± 15, preeclampsia+FGR 136 A.U. ± 13, healthy 146 A.U. ± 11, $P = 0.097$) (Fig. 2C).

Expression of HMGB1, TLR2 and TLR4 was also investigated in first trimester placentas to assess variations throughout gestation (Fig. S1). TLR2 (Fig. S1G) and

nuclear HMGB1 (Fig. S1C) were predominantly expressed in villous stromal cells and to a lesser extent in cytotrophoblasts, but not in the syncytium. TLR4 was strongly expressed in the first trimester syncytium and stromal cells (Fig. S1E).

HMGB1 pathway functionality in placental explants and trophoblasts

Functional response to the two HMGB1 isoforms was tested in third trimester placental explants (Fig. 3) and SGHPL-5 trophoblasts (Fig. 4). The cytokine HMGB1 isoform significantly increased the release of IL-6, IL-8, IFN-γ, TNF-α and VEGF-A from cultured chorionic villous explants after 24 h, while the chemokine HMGB1 isoform did not induce a cytokine response (Fig. 3A-E). Inhibition of TLR4-signaling confirmed a TLR4 dependent IL-8 response to the cytokine HMGB1 isoform (Fig. 3F).

The cytokine HMGB1 isoform also activated cultured SGHPL-5 trophoblasts to release significantly higher levels of IL-8 after 4 h (5-fold increase) and 24 h (3-fold increase), whereas the chemokine HMGB1 isoform did not influence IL-8 production (Fig. 4A,B). Inhibition of TLR4-signaling in SGHPL-5 cells confirmed that the IL-8 response to the cytokine HMGB1 isoform was TLR4-dependent (Fig. 4C). Release of IL-6, IFN-γ, TNF-α and VEGF-A by SGHPL-5 trophoblasts was not affected by HMGB1 (not shown).

Maternal serum levels of inflammatory and anti-angiogenic markers

Serum levels of HMGB1 were measured in non-pregnant women (n = 28), healthy pregnant women (gestational age 28-40 weeks) (n = 43) and preeclamptic women at delivery (gestational age 25-39 weeks) (n = 34). Maternal serum HMGB1 was significantly increased in preeclamptic (5.6 ng/ml [3.1 – 7.8]) and healthy pregnancies

(4.5 ng/ml [1.4 – 7.4]) compared to non-pregnant women (1.9 ng/ml [0.7 – 3.5], P = 0.0003) (Fig. 5A).

Maternal serum levels of HMGB1 correlated positively with maternal serum levels of the mainly placenta-derived preeclampsia marker sFlt-1 [44] (Spearman's r = 0.27, P = 0.005) and the inflammatory marker CRP (Spearman's r = 0.26, P = 0.008) (data not shown). The serum markers sFlt-1 and CRP were significantly increased in preeclampsia compared to normal pregnant and non-pregnant groups (Table 2).

Maternal serum IL-8 were significantly increased in preeclamptic (34 pg/ml [29 – 45]) compared to healthy pregnant women (24 pg/ml [20 – 33], P = 0.0001) (Fig. 5B).

Discussion

The pathway components HMGB1, TLR4 and IL-8 were here shown strongly co-expressed at the syncytium, while TLR2 was not detected. The syncytium TLR4 expression and maternal serum IL-8 were significantly increased in preeclampsia. Pathway functionality was confirmed by TLR4-dependent IL-8 release induced by the inflammatory isoform of HMGB1 in placental explants and trophoblasts. These data suggest involvement of isoform-specific HMGB1 activation of TLR4 in placental inflammation localized at the syncytium and a likely involvement in preeclampsia.
HMGB1, TLR4 and IL-8, but not TLR2, were all expressed in the syncytium in third trimester placentas, advocating that the syncytium responds to HMGB1 through TLR4 and not TLR2. The lack of syncytium TLR2 is supported by Ma *et al.* [34], while others have reported syncytium TLR2 staining [30, 32, 35]. This discrepancy could be explained by use of techniques to increase immunohistochemistry sensitivity or inadequate use of negative controls. The strong syncytium expression of TLR4 in early and late pregnancy is confirmed by others [30, 32, 34, 45], and highlights the importance of TLR4 at the maternal-fetal interface. Marked TLR4 expression on the syncytium side facing maternal blood [45] points to a protective function in normal pregnancy. In addition to TLR2 and TLR4, HMGB1 induces cytokine production through the receptor for advanced glycation end products (RAGE) [9]. Placental expression of RAGE has been shown to be increased in severe preeclampsia, but its expression and function needs to be further investigated to determine whether it functions as an additional placental receptor for HMGB1 [24, 32, 46]. Only nuclear HMGB1 was expressed in early pregnancy placentas, while the cytoplasmic HMGB1 expression that precedes its extracellular release was found in third trimester placentas,

pointing to HMGB1 as a more active mediator of placental inflammation at later gestation. To our knowledge, we are the first to assess the placental role of HMGB1 by cell-specific automated expression analysis of HMGB1 pathway components combined with functional HMGB1 isoform responses.

The increased TLR4 expression in preeclampsia and functional response to HMGB1 activation reported here substantiates a role in placental inflammation in preeclampsia, with the syncytium layer as the most important activation site. Since HMGB1 is abundantly expressed in placental cells and present in maternal serum, increased levels of its receptor TLR4 in the syncytium in preeclampsia is presumably sufficient for adverse activation. The increased syncytial TLR4 expression in preeclampsia is strongly supported [29, 30, 32, 47, 48] and may result from oxidative stress, a characteristic of the dysfunctional placenta in preeclampsia [1, 49]. TLR4 expression was increased in placentas from preeclamptic pregnancies despite their lower gestational age compared to controls, as it has been demonstrated that TLR4 activation and cytokine response increase with gestational age [50]. We did not adjust for the difference in gestational age and parity between groups since the difference is linked to the preeclampsia diagnosis and an adjustment may thus result in overadjustment bias, giving incorrect estimates of association [51]. The HMGB1 concentrations required to induce an in vitro TLR4 response were higher than the maternal serum HMGB1 levels. In vivo, the syncytium may be exposed to not only free serum HMGB1 but also to placental debris containing the danger signal, as well as HMGB1 released by the syncytium itself and by villous immune cells [21, 52]. Importantly, HMGB1 is not the only danger signal that can activate TLR4 in preeclampsia. Endogenous ligands for TLR4 are under intense investigation and may involve molecules such as advanced glycation end products,

oxidized lipoproteins and fibrinogen [10, 53]. Further examination is needed to reveal the complexity of danger signal involvement in preeclampsia.

The syncytium layer plays a central role in maternal-fetal interaction by shedding immunologically active components and cellular debris to the maternal circulation [48]. Whether or not syncytium HMGB1 and IL-8 expression is increased in preeclampsia still remains uncertain due to inconsistent findings by us and others [24, 31-33, 36, 54], warranting investigation in larger and more defined disease subgroups. Syncytium HMGB1 and IL-8 can be released to the placental extracellular environment and the maternal circulation. Increased maternal serum HMGB1 in preeclampsia has been reported [22, 24], but the results are controversial since others have not observed such increase [33]. This discrepancy is probably not due to the gestational variation between cases and controls observed in this study, because it has been shown that serum levels of HMGB1 do not correlate with gestational age [22, 33]. This highlights the need for HMGB1 isoform profiling in placental tissue and maternal serum to understand the role of functionally different HMGB1 isoforms in preeclampsia [13]. The current lack of isoform-specific antibodies for HMGB1 useful for immune assays complicates such investigations. Liquid chromatography-tandem mass spectrometry (LC/MS) may be used for future studies [52]. IL-8 has been found elevated in maternal serum by us and others [55-57], and may contribute to the systemic endothelial dysfunction in preeclampsia [55, 56, 58-60].

The inflammatory HMGB1-TLR4 pathway is shown at the syncytium as part of placental inflammation in normal third trimester pregnancies. By combined studies of pathway components, cellular players and functional responses, we have further indicated involvement of HMGB1-TLR4 in preeclampsia. In relation to the increase in

HMGB1 [22-24] and IL-8 in maternal serum in preeclampsia, this points to a potentially harmful interdependent communication between the placental surface and maternal blood in preeclampsia.

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Author contribution

K.C., A.L.B., R.B.S., L.C.V.T. and L.B. collected the clinical material and information. L.H.T., G.S.S. and A.C.I. conceived and designed the experiments. L.H.T., G.S.S., G.B.S., L.M.G. and B.S. performed the experiments and analyzed the data, and along with A.C.I. interpreted the data. L.H.T., G.B.S., G.S.S., L.B. and A.C.I. drafted the article. All authors critically revised the article and approved the final version.

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Declarations of interest: none

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Figures

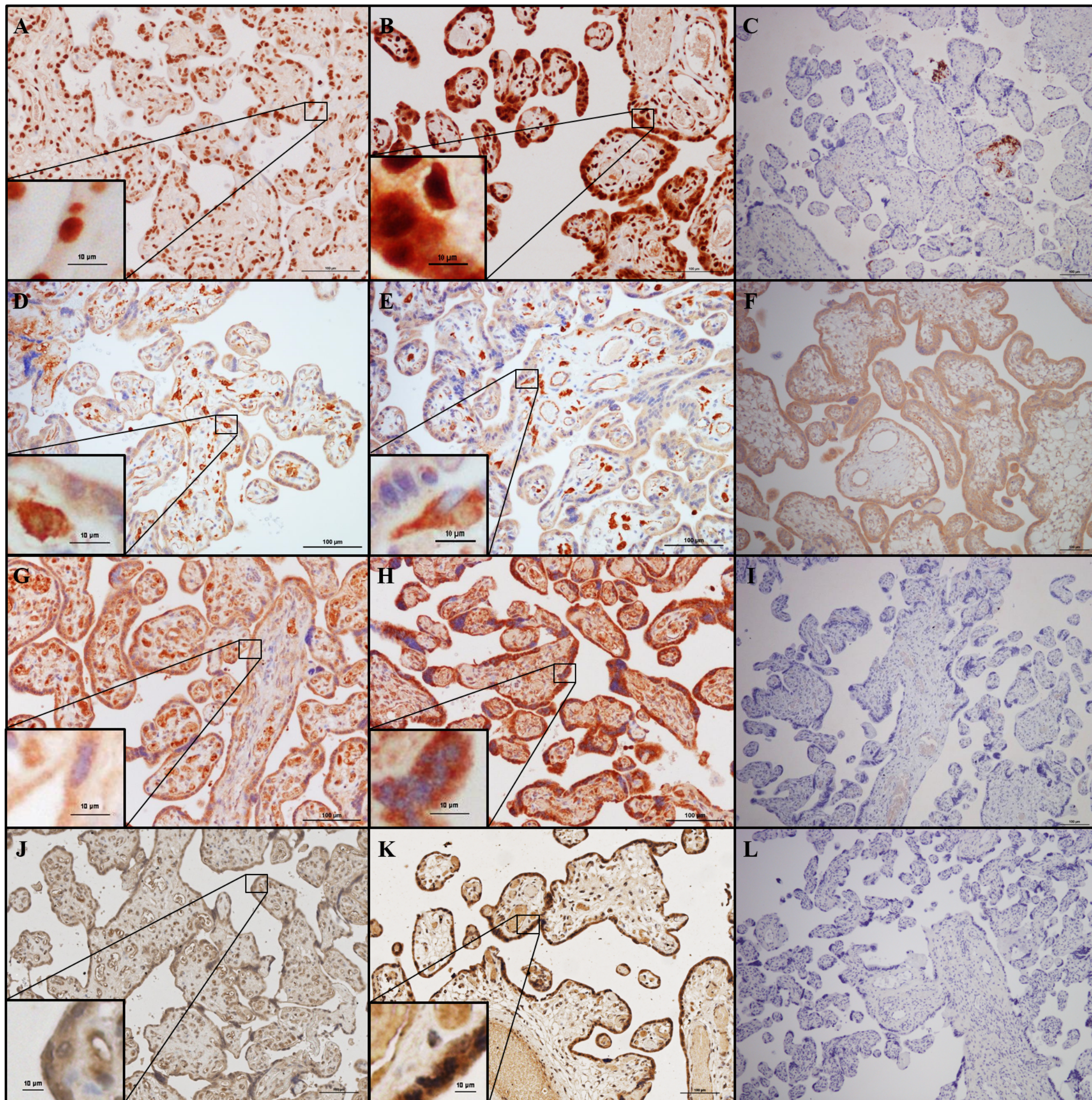

Fig. 1. HMGB1, TLR2, TLR4 and IL-8 expression in third trimester placenta. Third trimester placentas from healthy (n = 13) and preeclamptic (n = 23; of which 11 were without and 12 with fetal growth restriction (FGR)) pregnancies were stained for (A, B) high mobility group box 1 (HMGB1), (D, E) Toll-like receptor (TLR)2, (G, H) TLR4 and (J, K) interleukin (IL)-8. Representative images of (A, D, G, J) healthy placentas at gestational age 38+0 weeks and preeclamptic placentas at gestational age (B, H) 28+1, (E) 34+0 and (K) 29+0 weeks are shown. Isotype controls third trimester placentas (C,

F, I, L) were stained with isotype controls matching the specific antibodies used for (C) high mobility group box 1 (HMGB1), (F) Toll-like receptor (TLR)2, (I) TLR4, and (L) interleukin (IL)-8 staining.

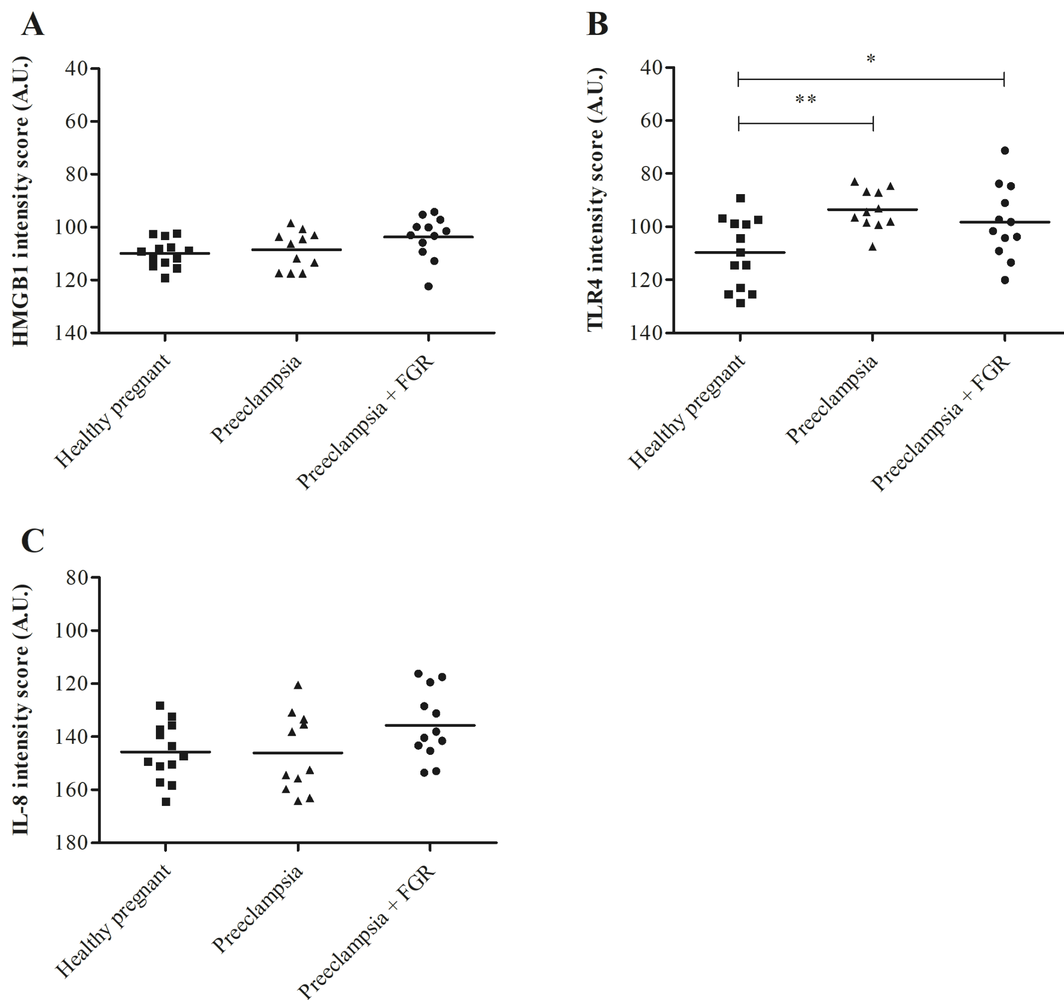

Fig. 2. Automated quantification of protein expression in third trimester placenta. For (A) HMGB1, (B) TLR4 and (C) IL-8 the syncytiotrophoblast staining was quantified by automated intensity analysis using NIS elements software, and data were analyzed using one-way ANOVA with Tukey's multiple comparison post-hoc test. $^{*}P < 0.05$. $^{**}P < 0.01$. A.U. indicates arbitrary units.

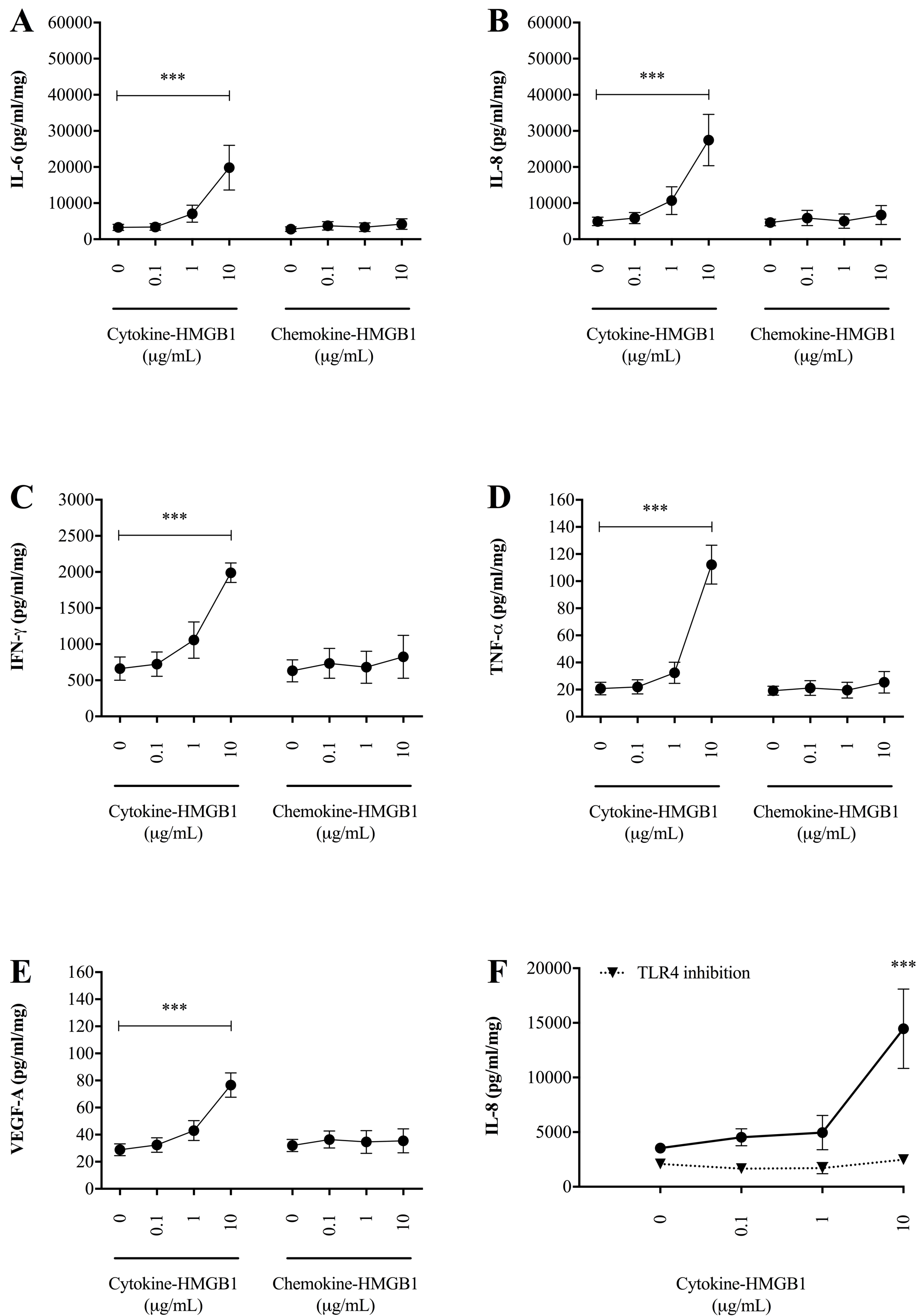

Fig. 3. HMGB1 activated cytokine response in third trimester placental explants. Third

trimester chorionic villous explants (n = 5) were incubated with either of the two isoforms of high mobility group box 1 (HMGB1) (0.1–10 µg/ml) for 24 h. Each of the eight experimental conditions was performed with six replicates (n = 48 explants per placenta). (A-E) Cytokines were quantified in supernatants by multiplex analysis. (F) TLR4 inhibition was performed in third trimester placental explants (n = 2) by incubation with the cytokine HMGB1 isoform (0.1–10 µg/ml) with or without the TLR4-inhibitior CLI-095 (10 µM) for 24 h, and IL-8 in supernatants was quantified by ELISA. Data are presented as mean ± SEM of six replicates from each of five (A-E) or two (F) independent experiments with cytokine levels relative to explant weight. Data were analyzed using one-way ANOVA with Dunnett's multiple comparison post-hoc test. ****P* < 0.001, compared to the (A-E) unstimulated or (F) TLR4 inhibited condition. IFN indicates interferon; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

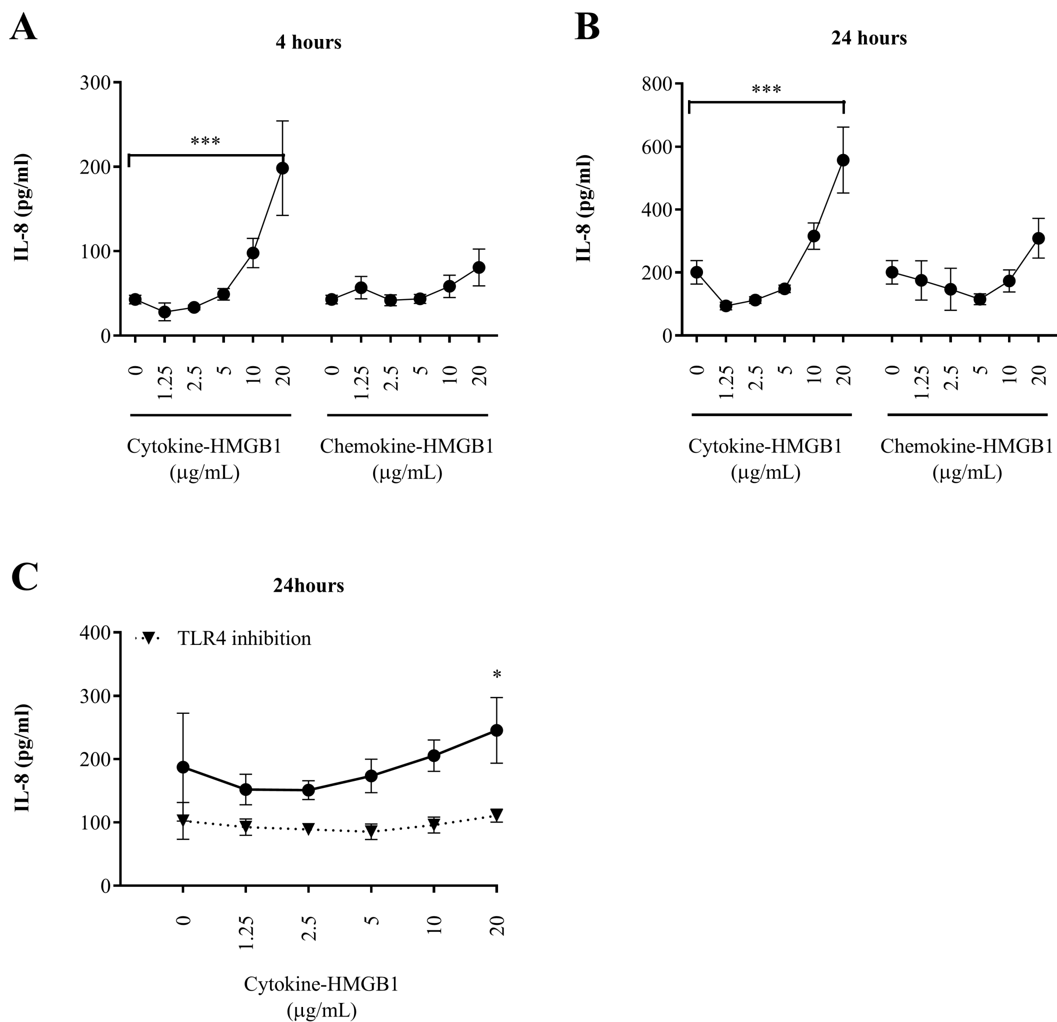

Fig. 4. HMGB1 activated IL-8 response in SGHPL-5 trophoblasts. The trophoblast cell line SGHPL-5 was incubated with either of the two isoforms of high mobility group box 1 (HMGB1) (1.25–20 µg/ml) for (A) 4 h or (B) 24 h. (A, B) Interleukin (IL)-8 was quantified in culture supernatant by multiplex analysis and is presented as mean ± SEM of triplicates from three independent experiments. (C) TLR4 inhibition was performed in SGHPL-5 trophoblasts (n = 2) by incubation with the cytokine HMGB1 isoform (1.25–20 µg/ml) with or without the TLR4-inhibitior CLI-095 (10 µM) for 24 h, and IL-8 in supernatants was quantified by ELISA. Data are presented as mean ± SEM analyzed using one-way ANOVA with Dunnett's multiple comparison post-hoc test. **P*

< 0.05, ***P < 0.001, compared to the (A,B) unstimulated or (C) TLR4 inhibited condition.

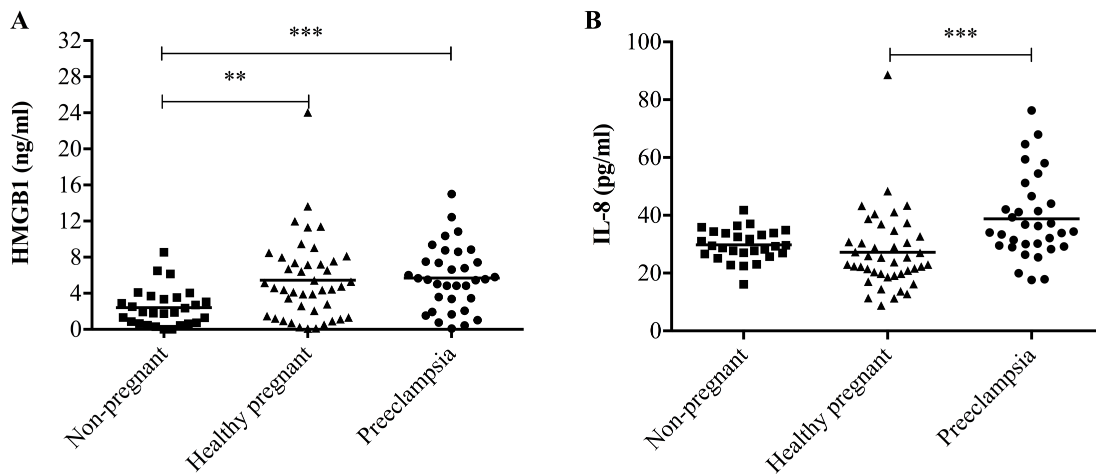

Fig. 5. Maternal serum levels of HMGB1 and IL-8. Serum levels of (A) high mobility group box 1 (HMGB1) and (B) interleukin (IL)-8 were measured in duplicate by ELISA in non-pregnant (n = 28), healthy pregnant (n = 43) and preeclamptic (n = 34) women. Data were analyzed using the Kruskal-Wallis test with Dunn's multiple comparison post-hoc test. **P < 0.01, ***P < 0.001.

Supplementary figures

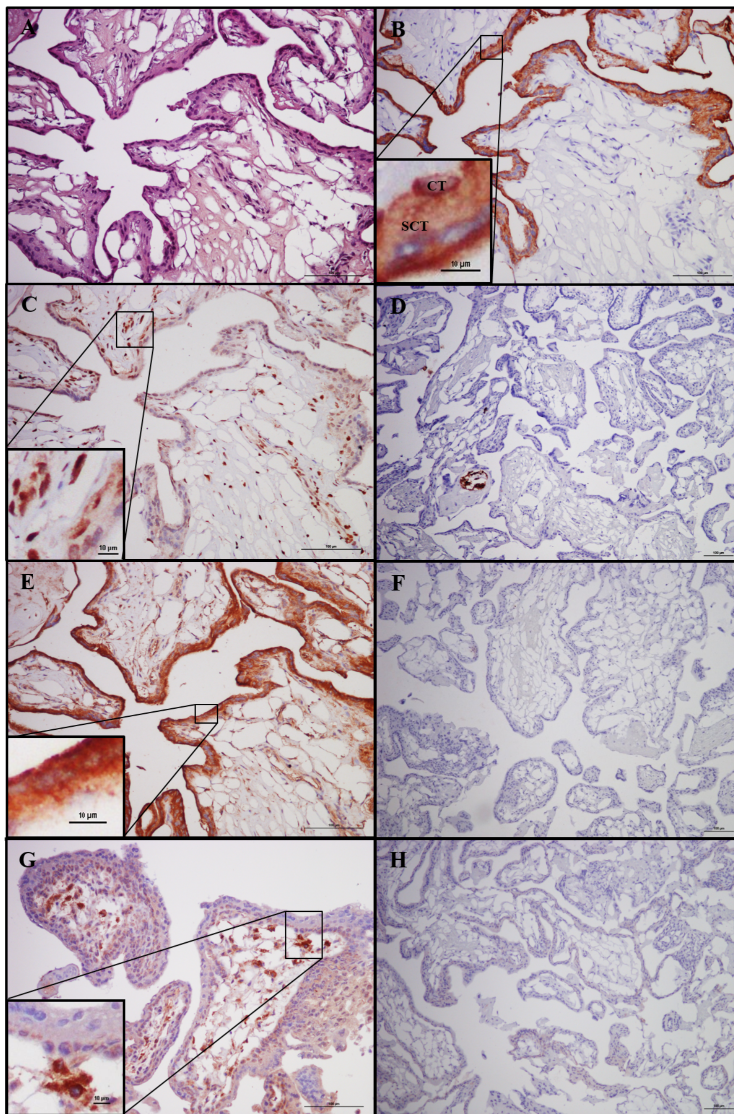

Fig. S1. HMGB1, TLR2 and TLR4 expression in first trimester placenta. First trimester placentas (n = 10) were stained for expression of (A) hematoxylin erythrosine saffron (HES), (B) the trophoblast marker cytokeratin (CK)7, (C) high mobility group box 1 (HMGB1), (E) Toll-like receptor (TLR) 4 and (G) TLR2. Representative images of a placenta at gestational age (G) 11+5 or (A-C, E) 12+3 weeks are shown. CT and SCT indicate cytotrophoblasts and syncytiotrophoblast. First trimester placentas were stained with isotype controls matching the specific antibodies used for (D) high mobility group box 1 (HMGB1), (F) Toll-like receptor (TLR) 4, (H) TLR2.

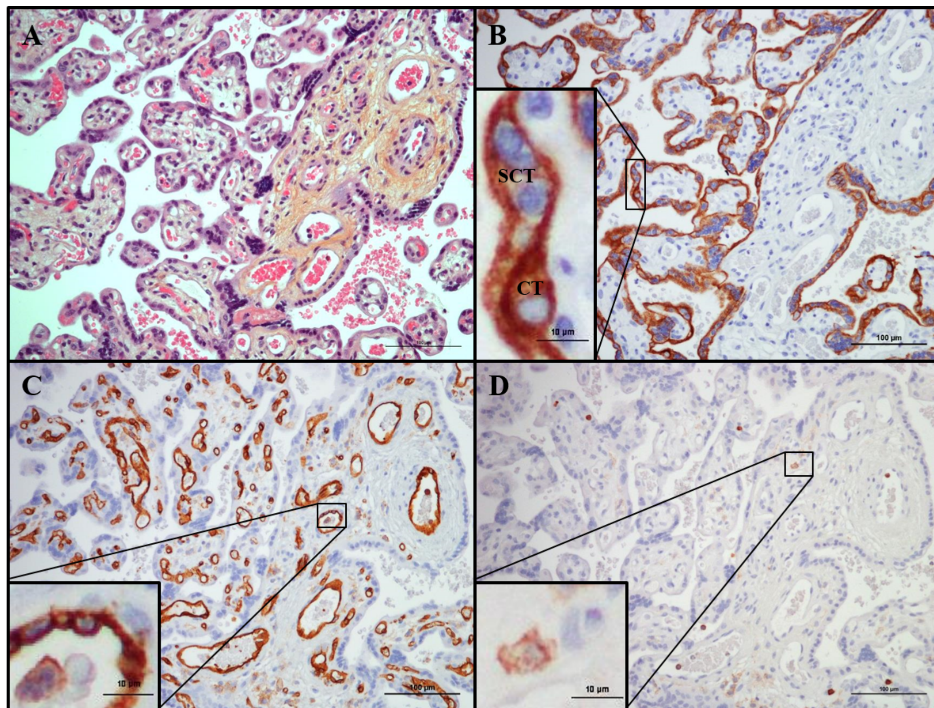

Fig. S2. HES staining and cell type specific markers in third trimester placenta. Third trimester placentas from healthy and preeclamptic pregnancies were stained by (A) hematoxylin erythrosine saffron (HES), and immunohistochemical staining for (B) the trophoblast marker cytokeratin 7 (CK7), (C) the endothelium marker CD31 and (D) the leukocyte marker CD45. Representative images of a healthy placenta at gestational age 38+0 weeks are shown. CT and SCT indicate cytotrophoblasts and syncytiotrophoblast.

Tables

Table 1. Clinical characteristics for subjects included in third trimester placental analyses

	Preeclampsia (n = 23)	**Healthy pregnant (n = 13)**
Baseline characteristics		
Maternal age (years)	30 (6)[a)]	32 (5)[a)]
Primiparae (*n*, %)	12 (52)[b),*]	1 (8)[b)]
Characteristics at delivery		
Body mass index (kg/m^2)	29 (8)[b)]	29 (6)[b)]
Systolic blood pressure (mmHg)	151 (12)[a),*]	119 (9)[a)]
Diastolic blood pressure (mmHg)	100 (8)[a),*]	71 (9)[a)]
Gestational age delivery (weeks)	32 (4)[b),*]	39 (1)[b)]
Placental weight (g)	350 (193)[b),*]	580 (241)[b)]
Fetal birth weight (g)	1780 (995)[b),*]	3590 (630)[b)]
FGR, (*n*, %)	12 (52)[b)]	n.a.
Severe preeclampsia[c)], (*n*, %)	16 (70)[b)]	n.a.

Continuous variables are presented as a) mean with standard deviation or b) median with interquartile range. FGR, fetal growth restriction; n.a.; not applicable. Comparisons Comparisons between outcome groups (two-sided T-tests or Mann-Whitney U-test for continuous variables, Chi-square test for categorical variables): *$P < 0.05$ vs healthy pregnant. c) Preeclampsia was sub-phenotyped as severe if diagnosed with one or more features [37, 61].

Table 2. Clinical characteristics and markers for subjects included in serum analyses

	Preeclampsia (n = 34)	Healthy pregnant (n = 43)	Non-pregnant (n = 28)
Baseline characteristics			
Maternal age (years)	31 (6)$^{a)}$	32 (4)$^{a)}$	30 (5)$^{a)}$
Primiparae (*n*, %)	19 (56)$^{b),*}$	4 (9)$^{b)}$	n.a.
Characteristics at time of phlebotomy			
Body mass index (kg/m^2)	28 (7)$^{b),**}$	28 (6)$^{b),**}$	22 (5)$^{b)}$
Systolic blood pressure (mmHg)	157 (22)$^{a),*,**}$	115 (13)$^{a)}$	111 (8)$^{a)}$
Diastolic blood pressure	99 (11)$^{a),*,**}$	69 (9)$^{a)}$	69 (7)$^{a)}$
GA at blood draw$^{c)}$ (weeks)	31 (4)$^{b),*}$	38 (11)$^{b)}$	n.a.
Characteristics at time of delivery			
GA delivery (weeks)	31 (4)$^{b),*}$	39 (1)$^{b)}$	n.a.
Placental weight (g)	280 (165)$^{b),*}$	620 (190)$^{b)}$	n.a.
Fetal birth weight (g)	1335 (876)$^{b),*}$	3540 (335)$^{b)}$	n.a.
FGR (*n*, %)	20 (59)$^{b)}$	n.a.	n.a.
Severe preeclampsia$^{d)}$ (*n*, %)	27 (79)$^{b)}$	n.a.	n.a.
Serum markers			
sFlt-1 (ng/ml)	1510 (1148)$^{b),*,**}$	154 (227)$^{b),**}$	0 (3)$^{b)}$
hsCRP (µg/ml)	4.5 (14.8)$^{b),*,**}$	3.1 (3.3)$^{b),**}$	0.5 (0.9)$^{b)}$

Continuous variables are presented as a) mean with standard deviation or b) median with interquartile range. FGR, fetal growth restriction; GA, gestational age; hsCRP, high sensitivity C-reactive protein; n.a., not applicable; sFlt, soluble fms-like tyrosine kinase. Comparisons between outcome groups (two-sided T-tests or Mann-Whitney U-test for comparisons of continuous variables between two groups, ANOVA and Tukey's test or Kruskal-Wallis and Dunn's test for comparisons of continuous variables between three groups Chi-square test for categorical variables): *$P < 0.05$ vs healthy pregnant.
**$P < 0.05$ vs healthy non-pregnant. c) GA at blood draw differs from GA at delivery only for healthy pregnant controls sampled during pregnancy (n = 18). d) Preeclampsia was sub-severe if diagnosed with one or more severe features [37, 61].