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DECREASED LEVELS OF FOLATE RECEPTOR-β AND REDUCED NUMBERS OF FETAL MACROPHAGES (HOFBAUER CELLS) IN PLACENTAS FROM PREGNANCIES WITH SEVERE PREECLAMPSIA (PE)*

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Abstract

Problem—Preeclampsia (PE), a pregnancy complication of unknown etiology, is a major cause of maternal and fetal mortality and morbidity. Previous studies have described placental genes which are up-regulated in expression in PE, but few studies have addressed placental gene suppression in this syndrome.

Method of Study—Gene profiling and quantitative reverse transcription PCR (qRTPCR) analyses were used to identify genes down-regulated in placentas from women with severe preterm PE compared to gestational age-matched normotensive controls with spontaneous preterm birth (sPTB). Western blotting and immunohistochemistry were used to evaluate levels and patterns of cell type-specific protein expression in PE and sPTB group placentas.

Results—Levels of macrophage marker [folate receptor (FR)-β, CD163, and CD68] mRNA and FR-β protein were significantly down-regulated in PE group placentas compared to the sPTB group. Numbers of Hofbauer cells (HBCs, fetal macrophages) and FR-β protein in these cells were reduced in PE group placentas.

Conclusion—Severe PE is associated with decreased placental expression of FR-β and a reduction in the number of HBCs. Reduced placental macrophage function is likely to play a key role in the pathophysiology of PE.

Keywords

Placenta; fetal macrophages; Hofbauer cells; folate receptor; preeclampsia

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INTRODUCTION

Preeclampsia (PE), characterized by pregnancy-induced maternal hypertension and proteinuria, complicates approximately 5% of all pregnancies, and remains a leading cause of maternal and fetal mortality and morbidity.[1] Although the precise etiology of PE is unknown, there is a consensus that failure to convert the maternal uterine vasculature to a high capacitance/low resistance network in the first trimester of pregnancy plays a key role.[2, 3] Impaired trophoblast invasion as well as decidual and immune cell dysfunction in PE are suggested to promote subsequent hypoxic/ischemic damage to placenta and aberrant activation of maternal vascular endothelia.[1–3] Placental damage has been reported to be most pronounced in pregnancies complicated by both PE and intrauterine growth restriction (IUGR).[4–6] Maternal blood levels of prostanoids, lipoperoxides, and inflammatory cytokines are elevated in PE, reflecting the pro-inflammatory nature of this condition.[7–9] Since clinical symptoms do not typically appear until after 20 weeks' gestation, it is extremely challenging to assess the role of individual factors in the pathophysiology of PE.

PE is associated with increased placental synthesis and release of proteins into the maternal circulation including the soluble anti-angiogenic factors, fms-like tyrosine kinase (sFlt-1) and endoglin (sEng),[10–14] which block binding of vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and transforming growth factor-β (TGF-β) to their cognate membrane receptors. It is suggested that increased placental levels of the anti-oxidant enzymes glutathione peroxidase and catalase serve a protective/adaptive function in response to a suboptimal intrauterine environment in PE.[15, 16] Similarly, we showed that levels of ceruloplasmin, an iron transport protein with anti-oxidant properties, are increased in placentas from pregnancies with PE and in trophoblast cultures in response to hypoxic treatment.[17]

Of note, chorionic villus sampling (CVS) at 10–12 weeks of gestation in patients who went on to develop PE identified 36 differentially expressed genes in PE, 31 of which were down-regulated.[18] Several genes were related to immunological processes, leading to the conclusion that fetal immune dysfunction occurs in the first trimester of pregnancies destined to develop PE. Similarly, genes specifically related to immune and hemostatic function including HLA-DRB4, claudin4, thrombin, and coagulation factor VIII were down-regulated in CVS samples from pregnancies which subsequently developed PE.[19, 20] Taken together, these studies provide a rationale for examination of down-regulated, potentially "non-compensatory" placental genes to provide further insights into placental dysfunction in PE.

To provide further insight into placental dysfunction in PE, in this study we used gene profiling and qRTPCR analysis to identify novel down-regulated genes in placentas from women with severe preterm PE compared to gestational age-matched normotensive control patients with spontaneous preterm birth (sPTB). Our results showed that severe PE was associated with decreased levels of genes expressed by Hofbauer cells (*i.e.* HBCs, placental fetal macrophages) as well as decreased levels of HBCs themselves. Our specific finding that PE was associated with decreased levels of folate receptor-β (FR-β), a critical folate carrier in macrophages,[21] is of note in light of the important role of folate in fetal growth and development.[22]

MATERIALS AND METHODS

Patient groups and tissue collection

We studied placental samples from 50 women enrolled in the following groups: severe PE (n=10); intrauterine growth restriction (IUGR) without PE (n=7); PE+IUGR (n=7); sPTB in

the absence of documented intra-amniotic inflammation (IAI) (n=11), and sPTB+IAI (n=9). A group of healthy term women with appropriately grown singleton fetuses undergoing cesarean delivery were also included as control (TC, n=6). Placentas were collected under protocols approved by the Human Investigation Committee of Yale University. Written informed consent was obtained from all participants, prior to enrollment. Gestational age was established based on menstrual date confirmed by sonographic examination prior to 20 weeks' gestation. Characteristics of the study population are shown in Table 1.

Only patients with severe PE (not mild PE) were included in the study. This definition was based on the American College of Obstetricians and Gynecologists (ACOG) criteria. All patients met the diagnosis of PE (gestational proteinuric hypertension) after >20 weeks' gestation, and had features of severe disease such as: (i) systolic blood pressure of ≥160 mm Hg and/or diastolic blood pressure of ≥110 mm Hg on 2 occasions at least 6 hours apart, (ii) proteinuria ≥5 g in a 24-hour urine specimen or ≥3+ on dipstick testing of 2 random urine samples collected at least 4 hours apart,[23] (iii) symptoms of cerebral or visual disturbances (headache, visual changes) or epigastric or right upper-quadrant pain, (iv) pulmonary edema or cyanosis, (v) oliguria (urinary output < 500 mL/24 h), (vi) elevated liver enzymes (≥ twice normal values), or (vii) thrombocytopenia (< 100,000 cells/μl). IUGR was defined as birth weight <10th percentile for gestational age at birth.

The sPTB group consisted of pregnancies with spontaneous preterm labor and/or preterm premature rupture of membranes (PPROM) without evidence of clinical chorioamnionitis (CAM). Clinical CAM was diagnosed by the presence of maternal fever (≥37.8°C), uterine tenderness, foul smelling amniotic fluid or visualization of pus at the time of the speculum examination, and maternal (>100 beats per minute) or fetal (>160 beats per minute) tachycardia.[24, 25] All but one of the patients in the sPTB+IAI group had a clinically-indicated amniocentesis to rule out or confirm microbial invasion of the amniotic cavity. The remaining case presented with clinical CAM for which delivery was indicated. Histological examination of the placentas was performed by a certified perinatal pathologist, unaware of the clinical presentation or outcome. From each placenta, sections of chorionic plate, fetal membranes, and umbilical cord were examined for inflammation. Three histologic stages of CAM (stage I: intervillositis, stage II: chorionic inflammation, and stage III: full-thickness inflammation of both chorion and amnion) were assigned based on a histologic grading system.[26] The majority of patients in the preterm groups were administered antenatal corticosteroids in compliance with previous clinical recommendations.[27]

Following delivery, placentas were brought immediately to the laboratory and the decidua basalis layer from a central cotyledon, noted to be free of macroscopic pathology (*i.e.* fibrin deposition or infarcts), was dissected and discarded. Approximately 1 g of underlying villous tissue was collected, frozen in liquid nitrogen, and maintained at −80°C for use in gene array, qRTPCR, and Western blot studies. In addition, full thickness placental tissue specimens from both PE (n=10) and sPTB groups (n=11) were fixed in formalin for immunohistochemical analysis as described below.

DNA microarray analysis

RNA was extracted from snap frozen placental samples from pregnancies with both PE and IUGR as well as sPTB (n=3 different placentas for each experimental group) using Trizol reagent (Sigma-Aldrich, St. Louis, MO). RNA was then purified using the RNeasy Mini Kit (Qiagen, Germantown, MD), and 20 μg of purified RNA was submitted to the Keck Facility at Yale University School of Medicine for analysis of gene expression using the full human genome chip (HG_U133 Plus 2.0, Affymetrix, Santa Clara, CA) containing approximately 54K transcripts. Gene expression was analyzed using GCOS 1.4 GeneSpring (Affymetrix)

and GX 7.3.1 (Agilent, San Jose, CA) software. A 2-fold cut-off was used to establish an increase in gene expression as we have previously described.[17]

Quantitative reverse transcription PCR (qRTPCR)

Levels of mRNA were determined with an ABI 7500 RealTime PCR System (Applied Biosystems, Foster City, CA) using TaqMan Gene Expression Assays for FR-β (Cat# 00265255), CD163 (Cat# 01016661), CD68 (Cat# 00154355), CD206 (Cat# 00267207), CD209 (Cat# 01588349), Flt-1(Cat# 0152937), endoglin (Cat# 00164438), leptin (Cat# 00174977), and 18S RNA (Cat# 99999901). PCR reactions were performed in duplicate in a 20 μl volume of TaqMan Universal PCR Master Mix containing 1 μl of reverse transcription cDNA and 1 μl of assay primer-probe mix. Mean C_t values were analyzed using the 7500 System SDS software 1.3.1. Gene expression was normalized to the housekeeping gene 18S using the formula $2^{-\Delta\Delta Ct}$. Results are expressed as relative expression compared to an endogenous control in each experiment as we have previously described.[28]

Immunohistochemistry

Five μm placental villous tissue sections were deparaffinized at 58°C overnight, washed 3 times with xylene, and progressively rehydrated to 70% ethanol and PBS. Antigen retrieval was achieved by microwaving for 20 min in 10 mM sodium citrate buffer, pH 6.0. Sections were blocked with10% donkey serum and were incubated overnight at 4°C with sheep anti-FR-β (Cat# AF5697, R&D Systems, Minneapolis, MN), mouse anti-CD163 (Cat# MCA1853, AbD Serotec, Raleigh NC), and mouse anti-CD68 (Cat# M0814, Dako, Carpinteria, CA) primary antibodies, all at 1:100 dilution. Mouse IgG (Cat# X0931, Dako) and Sheep IgG (Cat# 013000003, Jackson ImmunoResearch, West Grove, PA) served as negative controls. The following day, endogenous peroxidase was quenched with 3% hydrogen peroxide in 50% methanol for 12 min, and after washing in PBS, biotinylated donkey anti-mouse (Cat# 715065151, Jackson ImmunoResearch) or anti-sheep (Cat# 713065003, Jackson ImmunoResearch) secondary antibodies were added for 30 min at a dilution of 1:1000. Staining was developed using the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA) and diaminobenzidine substrate. Slides were counterstained with hematoxylin, dehydrated to 100% ethanol and then xylene, and were mounted with CytoSeal 60. Visualization and photography was conducted with an IX71 inverted microscope (Olympus, Melville, NY). The number of cells positive for CD163, FR-β, and CD68 in serial sections were quantitated from pictures of 20 random, well-spaced 20× fields in 11 sPTB and 10 PE group placentas. Cells were counted from captured images using Image J software and results are presented as total number of positive cells per 20 fields. H-score analysis was carried out to analyze FR-β intensity semi-quantitatively in 5 sPTB and 6 PE group placentas using AxioVision and the corresponding digital image processing software (Carl Zeiss Microimaging, Inc, Thornwood, New York) according to the following scale: 0+ (no staining), 1+ (weak but detectable staining), 2+ (moderate staining), or 3+ (intense staining). For each slide, a computer-generated Histologic score (H-score) value was determined as previously described[29, 30] by calculating the sum of the number of cells that stain at each intensity scale and multiplying that value by the weighted intensity scale using the following formula: H-score = $\Sigma\pi$ (i+1), where "i" is the intensity scale and the "π" is the percentage of cells staining at the scale. For each slide, 5 fields and at least 100 cells per area were evaluated under a light microscope using 400× magnification. Results are reported as mean + standard error (SE) from a minimum of 5 separate readings from 3 separate tissue sections.

Western blotting

Protein was extracted from frozen placental tissue by homogenization in ProteoJet cell lysis buffer (Fermentas Life Sciences, Hanover, MD) supplemented with Compleat protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) using a ratio of 0.1 g tissue/1 ml of lysis buffer. Protein was quantitated by DC Protein Assay (BioRad Laboratories, Hercules, CA). Protein was run on a Tris-HCl 4–15% SDS PAGE gel (BioRad) then transferred to a nitrocellulose membrane. Following transfer, the membrane was incubated for 1 h at room temperature in Odyssey blocking buffer, and then overnight at 4°C with primary antibody in PBS containing 0.1%Tween (TPBS) with 5% powdered milk. Sheep anti-FR-β (see above) and goat anti-HSP90 (housekeeping protein, Cat# AF3286, R&D Systems) primary antibodies were all used at 1:2,000 dilution. IR-Dye800CW conjugated donkey anti-sheep (Cat# 613731168, Rockland, Gilbertsville, PA), and donkey anti-goat (Cat# 605731125, Rockland) secondary antibodies were all used at a 1:10,000 dilution. Target and housekeeping proteins were detected simultaneously on the same membrane. The membrane was washed in TPBS, and secondary antibodies were added for 45 min at room temperature. Following washing fluorophores were visualized and quantitated with an Odyssey Infrared Imager (LI-COR, Lincoln, NE) as we have previously described.[31]

Statistical analysis

Data sets were subjected to normality testing using the Kolmogorov-Smirnov method as previously carried out by our group.[17] Demographic, clinical, and laboratory characteristics are reported as a mean ± SE. Most comparisons between groups were performed using one-way analysis of variance (ANOVA). Kruskal-Wallis ANOVA was carried out for data that were not normally distributed and are presented as a median with quartiles. Proportions were compared with Chi square tests. Differences in placental mRNA and protein expression between patient groups measured by qRTPCR and Western blotting were analyzed by Kruskal-Wallis ANOVA, Mann-Whitney rank sum, and Student's t test. SigmaStat software was used for statistical analyses. A $P < 0.05$ was considered significant in all studies.

RESULTS

Decreased levels of macrophage-associated mRNA and protein in placentas from pregnancies with severe preterm PE

The characteristics of the six groups in the study population are shown in Table 1. Gestational age at delivery was significantly earlier in the severe PE (n=10), IUGR (n=7), PE+IUGR (n=7), and sPTB+IAI (n=9) groups compared to term control (TC, n=6, $P < 0.001$), but not significantly different than that of the sPTB group (n=11). Initial studies using gene array analysis revealed that pregnancies complicated by preterm PE+IUGR were associated with a 2.6-fold reduction in levels of placental FR-β mRNA compared to the sPTB group (n=3 per group, $P<0.05$). Since FR-β is a major folate transporter in macrophages,[21] we then used qRTPCR to compare expression of mRNA for several macrophage proteins in placentas from PE (n=10) and sPTB groups (n=11) (Fig. 1A). We observed that expression of FR-β and CD163 were significantly reduced 73% and 79% respectively in the PE group compared to the sPTB group. Levels of CD68 expression were reduced 59% in the PE group, but this effect did not reach statistical significance. In addition, mRNA levels of macrophage markers CD206 and CD209 were similarly down-regulated 1.8- and 4.3-fold in PE group placentas (not shown). These results indicate coordinate down-regulation of macrophage gene expression in PE placentas. Conversely, and consistent with prior publications,[14, 32, 33] levels of endoglin, Flt-1, and leptin mRNA were up-regulated by 2.4-, 5.4-, and 310-fold, respectively in PE group placentas (Fig. 1B).

Of note, qRTPCR revealed that levels of FR-β mRNA was significantly down-regulated 85% in pregnancies complicated by both PE+IUGR (n=7) compared to sPTB (n=11), whereas levels of these placental mRNAs were not significantly different in IUGR (n=7), sPTB+IAI (n=9), and term (n=6) groups compared to the sPTB group (Fig. 2). These results indicated that macrophage gene expression was selectively down-regulated in pregnancies with severe PE. Western blotting revealed that FR-β protein levels were significantly down-regulated by 65% in the PE group (Fig. 3), but not in pregnancies complicated by IUGR or sPTB+IAI (Fig. 4).

Decreased numbers of HBCs in placental villi in pregnancies with PE

Immunohistochemistry localized CD163, FR-β, and CD68 to select cells within the villus core (i.e. the same location of HBCs in placenta) (Fig. 5, Panels A to H). Staining for CD68 staining was relatively low in the stroma, but present in syncytiotrophoblast and endothelial cells (Fig. 5, Panels I to L). Non-macrophage staining in the placenta has been noted by other groups using several different CD68 antibodies.[34, 35] No staining was observed when isotype-matched control antibodies were substituted for primary antibodies (Fig. 5, Panels M and N). Quantitation of immunohistochemistry results as described revealed significant 28%, 34%, and 58% decreases in the numbers of FR-β^+, CD163$^+$, and CD68$^+$ cells in the placental stroma of the PE group compared to the sPTB group (Fig. 6A). H-score analysis, which combines measurement of cell number and intensity of staining, revealed a significant 45% decrease in levels of FR-β^+ cells in the villous stroma in PE group placentas (Fig. 6B). Taken together, these results indicate that severe PE is associated with a decrease in placental levels of macrophage marker mRNA and protein as well as a reduction in the number of HBCs.

DISCUSSION

To gain further insight into early placental gene dysregulation in PE, gene array analysis and qRTPCR was initially employed to examine mRNAs which were down-regulated in PE placentas compared to preterm gestational age-matched “control” (i.e. sPTB) placentas, as well as normal placentas at term. Our results indicated that placental expression of FR-β, CD163, and CD68 (i.e. macrophage markers) was specifically down-regulated in patients with severe preterm PE. Our demonstration of enhanced expression of endoglin, Flt-1, and leptin mRNA in PE group placentas by gene profiling and qRTPCR confirms the biological significance of our new findings since all of these mRNAs were previously demonstrated to increase in placentas from patients with PE.[14, 32, 33] Similarly, Western blotting revealed decreased levels of FR-β protein in PE group placentas. Immunohistochemistry revealed that macrophage markers were localized to HBCs, described in placental villi as large (10–30 μm), pleiomorphic, and highly vacuolated cells with a granular cytoplasm located in the placental stroma, which is below the syncytium and above fetal capillaries.[36, 37] HBCs first appear on the 18th day of gestation and are found until term.[38] Our immunohistochemical results indicated that PE was associated with a 28%–40% reduction in the number of placental cells expressing macrophage markers (i.e. HBCs). Importantly, qRTPCR and Western blotting revealed that PE was also associated with approximately an 80% reduction in FR-β and CD163 mRNA and FR-β protein levels, suggesting that HBC numbers and gene expression in HBCs are both down-regulated in pregnancies with PE. FR-β and CD163 are key regulators of macrophage function as they mediate folate transport and scavenging of free hemoglobin, respectively.[21, 39] There are two major plasma membrane-associated FRs: FR-α, which is expressed by epithelial cells, and FR-β which is specific for the cells of the monocyte/macrophage lineage.[21] Our results are consistent with recent observations for complement C3a receptor, which was localized to HBCs, and found to be expressed at lower levels in placentas from pregnancies with severe PE compared to controls.[40]

In the current study, as well as in previous immunohistochemical studies,[41] HBCs were observed to express anti-inflammatory M2 macrophage markers (i.e. FR-β and CD163), the expression of which was down-regulated in PE. This suggests that HBCs promote anti-inflammatory, pro-angiogenic responses in placenta. Consistent with this idea, immunohistochemical analysis in first trimester placentas indicated that HBCs were in close contact with endothelial progenitor cells and primitive vessels.[42] HBCs expressed VEGF at higher levels than peritoneal macrophages,[43] and sprouty proteins, regulators of branching morphogenesis, were localized to HBCs in placental tissue by double-immunofluorescence microscopy.[44] A recent study using mixed placental stromal cell cultures showed significant plasticity of HBC morphology and paracrine support of fibroblasts, suggesting that they may promote the maturation and development of the placental mesenchyme.[45] Based on the known plasticity and polarization of macrophages,[46] it is also feasible that PE is associated with a change in HBC phenotype and function toward the M1/pro-inflammatory macrophage continuum.

Changes in the number or appearance of HBCs have been associated with several complications of pregnancy.[47] Of note, the use of Y-chromosome markers demonstrated that increased numbers of HBCs in placenta in villitis of unknown etiology (VUE), a destructive inflammatory lesion of the chorionic villi akin to graft rejection, were derived from fetal precursors.[48, 49] In contrast to VUE, CAM is most often caused by ascending genital tract microorganisms that promote infiltration of neutrophils in maternal decidua and fetal membranes.[50] In our recent study, we noted a 3-fold focal increase in HBCs in the placental villus in CAM.[51] This is consistent with findings of another group demonstrating increased infiltration of $CD68^+$ cells in the placental villous stroma and fetal membrane choriodecidua in pregnancies complicated by CAM delivered at term.[52] Conversely, it was reported that $CD68^+$ cells decrease in the placental villus in association with advancing gestational age and CAM.[53] In this study, $CD68^+$ cells in an entire villous area were quantitated by computerized imaging. Since HBCs are not homogenously distributed in placental villi,[38, 54] we quantitated only those areas in which we observed one or more macrophages in terminal villi and five or more in intermediate villi. This indicates that term placentas show focal increases in numbers of HBCs in pregnancies with CAM even if the total numbers of villous $CD68^+$ cells may decrease or not change.

Functions of HBCs, like other tissue macrophages, include phagocytosis and scavenging, and regulation of immune cell function through the release of pro- and anti-inflammatory cytokines. PE is known to be associated with increased apoptosis of placental cytotrophoblasts and syncytiotrophoblasts.[55–57] Decreased HBC-mediated scavenging of apoptotic bodies in PE may lead to higher levels of placental damage and increased release of cellular debris and microparticles to maternal blood observed in PE [58–61]. Similarly, levels of free hemoglobin, a highly cytotoxic compound, noted to be elevated in placenta and maternal blood in PE,[62, 63] may in part be due to a loss of CD163-dependent scavenging of heme by HBCs. The PE-associated reduction in the numbers of HBCs and/or down-regulation of FR-β levels in HBCs could also compromise folate uptake by placenta. FRs are the major mediators of cellular uptake of folate when serum concentrations of folate are low.[21] Thus, folate deficiency may be expected to exacerbate placental damage and dysfunction noted in PE.[6, 64] Several studies also support an anti-inflammatory/stabilizing function for HBCs.[65–67] Cultures of HBCs released prostaglandin E_2,supported trophoblast function,[65, 67] and suppressed T cell responses.[66] Thus, reduced numbers of HBCs in patients with severe preterm PE may play an important role in promoting the pro-inflammatory, anti-angiogenic processes characteristically observed in placentas delivered from patients with this pregnancy complication.[1, 14, 64] To date, there is no information available concerning alterations in placental folate transport associated with PE.

In conclusion, our results demonstrate that severe PE is associated with a reduction in the number of HBCs as well as a decrease in placental levels of FR-β and other key HBC proteins. HBC dysfunction may promote the PE-associated disruption of placental architecture and the release of factors deleterious to maternal health.

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Abbreviations

CAM	chorioamnionitis
CVS	chorionic villus sampling
FR-β	folate receptor-β
HELLP	hemolysis, elevated liver enzymes, low platelet count
IAI	intra-amniotic inflammation
IUGR	intrauterine growth restriction
PE	preeclampsia
PlGF	placental growth factor
qRTPCR	quantitative reverse transcription PCR
sEng	soluble endoglin
sFlt-1	soluble fms-like tyrosine kinase-1
sPTB	spontaneous preterm birth
TC	term control
TGF-β	transforming growth factor-β
VEGF	vascular endothelial growth factor

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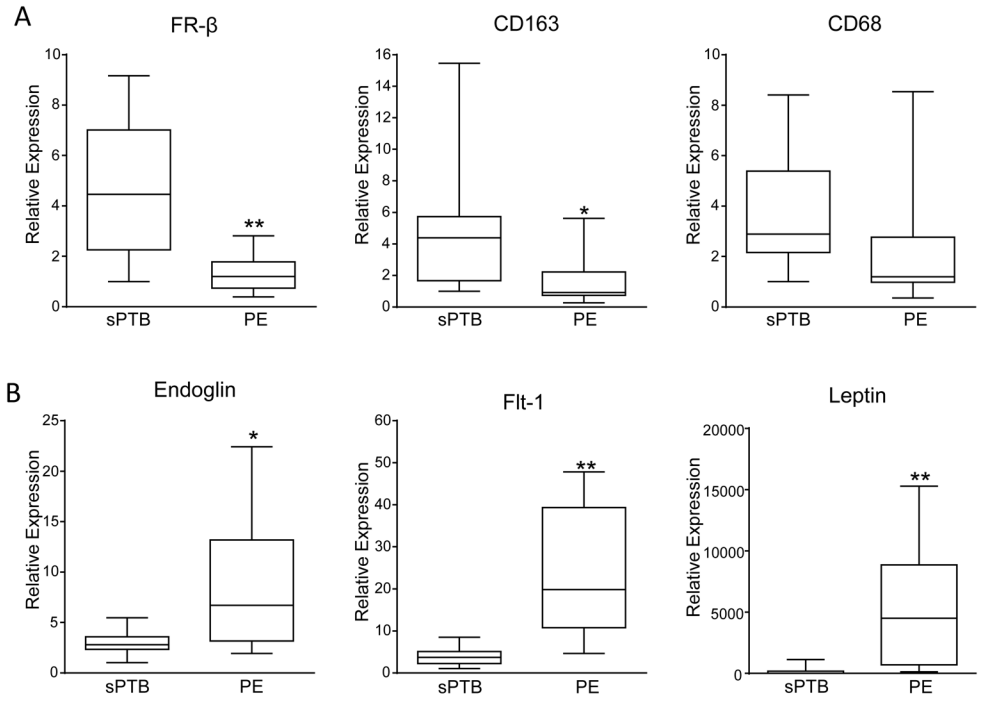

Figure 1. Analysis of placental gene expression by qRTPCR

RNA was extracted from whole placental tissue from severe preeclampsia (PE, n=10) and spontaneous preterm birth (sPTB, n=11) groups and converted to cDNA. Levels of macrophage marker gene expression (A) and genes known to be induced in PE (B) were then determined by qRTPCR and normalized to levels of 18S RNA. Results (not normally distributed) are presented as medians and percentiles. The lines inside the box indicate the median, the ends of the box describe the lower and upper quartiles, and the whiskers define the smallest and largest values.

*P<0.05 vs sPTB; **P<0.001 vs sPTB

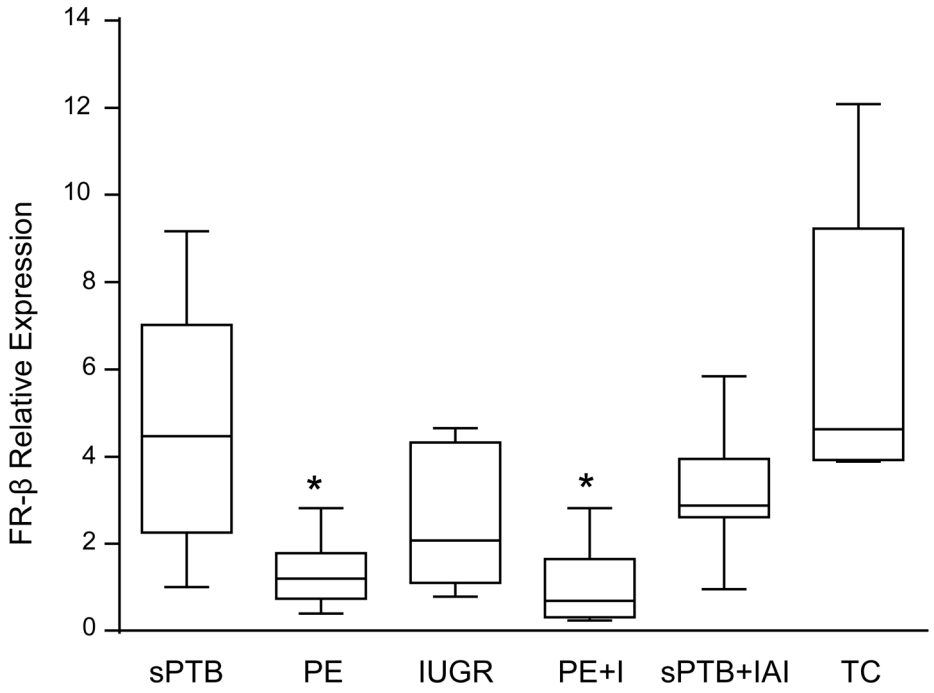

Figure 2. Analysis of placental FR-β gene expression by qRTPCR

RNA was extracted from whole placental tissue from sPTB (n=10), PE (n=11), IUGR (n=7), PE+IUGR (PE+I, n=7), sPTB+IAI (n=9), and TC (n=6) groups and converted to cDNA. Levels of FR-β mRNA expression were then determined by qRTPCR and normalized to levels of 18S RNA. Results are presented as medians and percentiles. The lines inside the box indicate the median, the ends of the box describe the lower and upper quartiles, and the whiskers define the smallest and largest values.

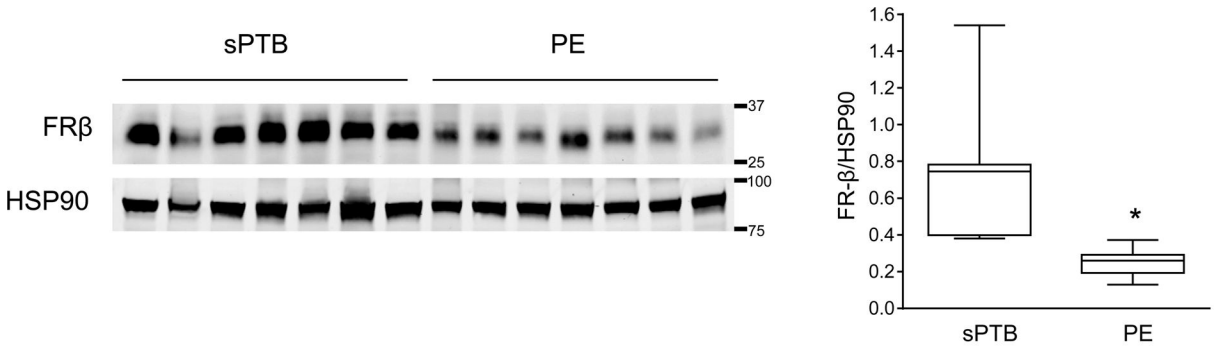

Figure 3. Measurement of placental FR-β protein levels in PE and sPTB group placentas by Western blotting

Western blot analysis of whole placental tissue extracts was carried out to assess the relative levels of FR-β in PE and sPTB groups (n=7 different placentas per group). FR-β was detected at a molecular weight of approximately 30 kDa (Left Panel, Top Blot). The migration of molecular weight standards is shown at the right of the blot. HSP90 served as a housekeeping protein control (Bottom Blot). Quantitation of FR-β expression is presented in the right panel. Results are presented as a median, lower and upper quartiles, and smallest and largest values.

*P<0.001 vs sPTB

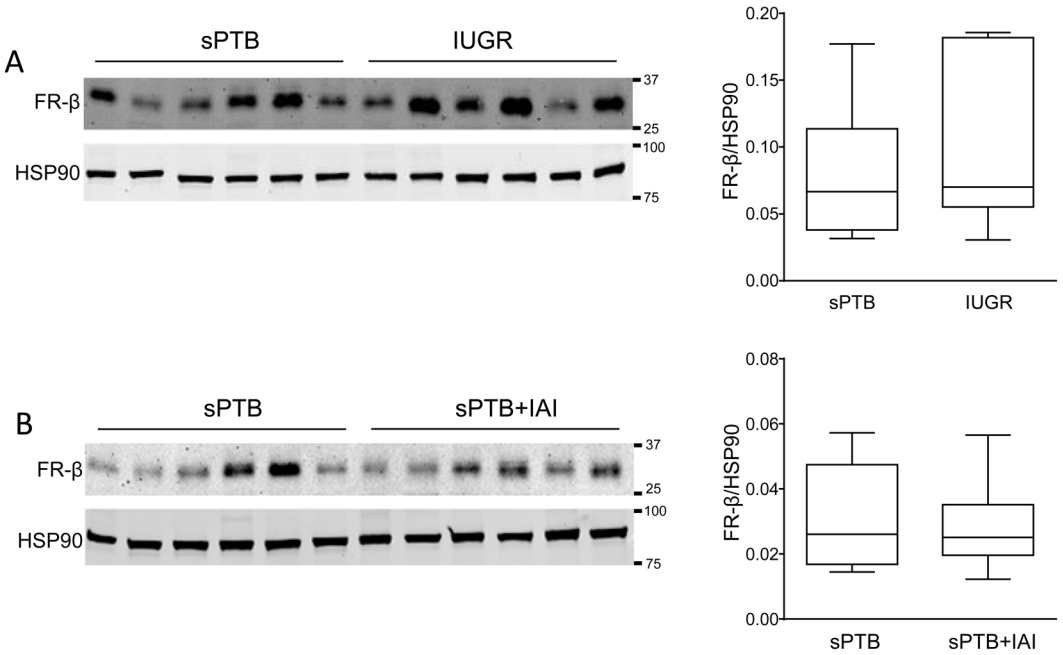

Figure 4. Measurement of placental FR-β protein levels in sPTB, IUGR, and sPTB+IAI groups by Western blotting

Western blot analysis of whole placental tissue extracts was carried out to assess the relative levels of FR-β in sPTB, IUGR, and sPTB+IAI groups (n=7 different placentas per group). Quantitation of FR-β protein in sPTB vs IUGR (A) and sPTB vs sPTB+IAI (B) groups was performed following normalization to HSP90 expression. Results are presented as a median, lower and upper quartiles, and smallest and largest values.

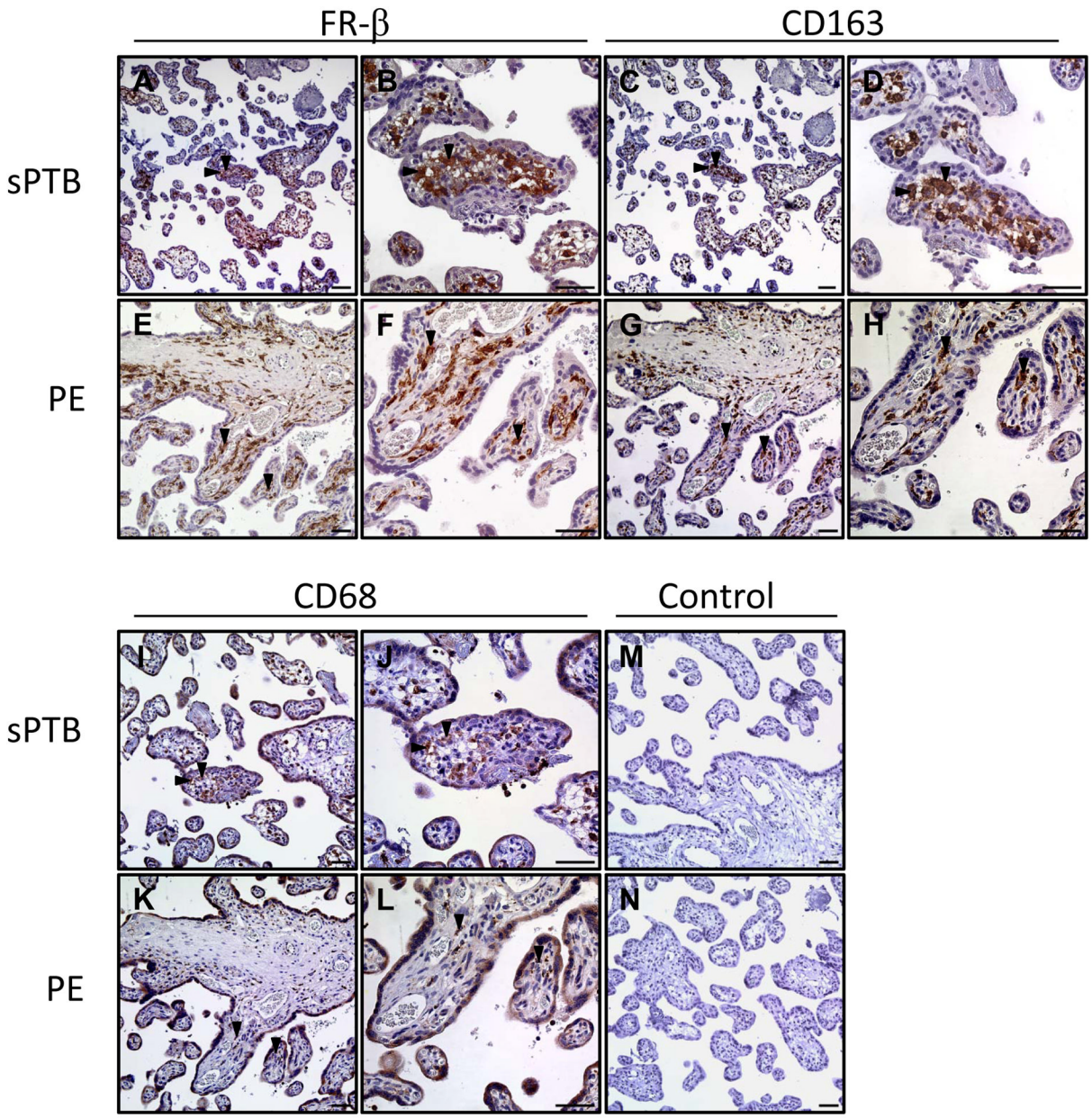

Figure 5. Immunohistochemical detection of macrophage markers in sPTB and PE placentas

Immunohistochemistry was used to localize FR-β, CD163, and CD68 in villi from sPTB (Panels A, C, and I) and PE (Panels and E, G, and K) group placentas (200× magnification). Higher magnification (400×) micrographs for the sPTB (Panels B, D, and J) and PE (Panels F, H, and L) are also shown. Micrographs are presented from a single placenta per group, representing immunohistochemical analysis of 11 sPTB and 10 PE group placentas. Arrowheads indicate brown diaminobenzidine staining for the macrophage markers. No staining was observed when non-specific mouse IgG replaced the primary antibody (Panels M and N). Bar=50 μm.

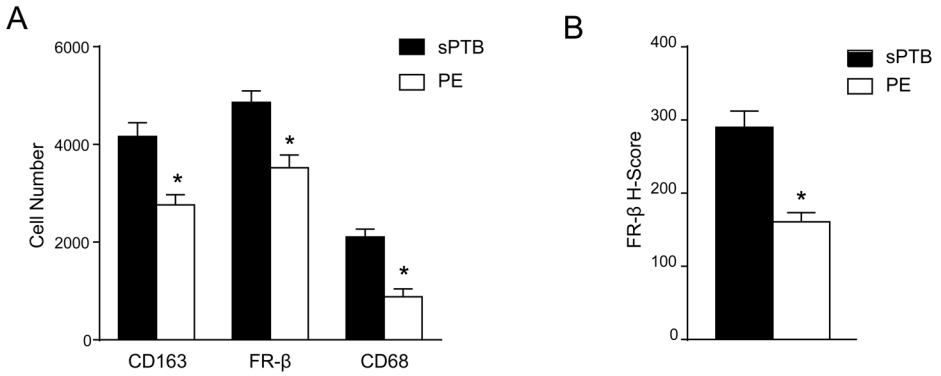

Figure 6. Quantitation of immunohistochemical results for macrophage markers in sPTB and PE group placentas

The number of cells positive for CD163, FR-β, and CD68 was compared in sPTB (n=11) and PE (n=10) group placentas (Panel A). H-score analysis was also carried out to assess FR-β staining in sPTB (n=5) and PE group (n=6) placentas (Panel B). Results for both measurements are expressed as a mean + SE.

*P<0.001 vs sPTB

Table 1

Characteristics of the study population at delivery (n = 50)

	PE n = 10	IUGR n = 7	PE+IUGR n = 7	sPTB+IAI n = 9	sPTB n = 11	TC n = 6	*P* value
Demographic and clinical characteristics							
Age, years *	29 ± 2	28 ± 3	26 ± 4	29 ± 1	24 ± 2	30 ± 3	0.451
Nulliparity †	9 (90)	3 (43)	5 (71)	1 (11)	7 (64)	0 (0)	0.001
Gestational age, weeks *	30 ± 1	31 ± 1	28 ± 1	26 ± 1	30 ± 1	39 ± 1	<0.001
PPROM †	0 (0)	0 (0)	0 (0)	4 (44)	6 (55)	0 (0)	0.002
Clinical chorioamnionitis †	0 (0)	0 (0)	0 (0)	3 (33)	0 (0)	0 (0)	0.052
Neurological symptoms †	5 (50)	0 (0)	2 (29)	0 (0)	0 (0)	0 (0)	0.002
Systolic blood pressure, mmHg *	165 ± 3	123 ± 8	160 ± 5	104 ± 4	113 ± 5	119 ± 4	<0.001
Diastolic blood pressure, mmHg *	104 ± 2	77 ± 6	92 ± 2	64 ± 4	60 ± 3	67 ± 6	<0.001
HELLP syndrome †	4 (40)	0 (0)	1 (14)	0 (0)	0 (0)	0 (0)	0.017
Antenatal steroids †	9 (90)	5 (71)	6 (86)	9 (100)	8 (73)	0 (0)	0.001
Laboratory characteristics							
Positive amniotic fluid cultures †	NA	NA	NA	8 (89)	0 (0)	NA	<0.001
Dipstick protein ‡	4 [3–4]	0 [0-0]	2 [2–3]	0 [0-0]	0 [0-0]	0 [0-0]	<0.001
24 hour protein excretion, grams *	3.7 ± 0.5	0.1 ± 0.0	1.3 ± 0.5	NA	NA	NA	<0.001
Outcome characteristics							
Birthweight, grams *	1,266 ± 147	959 ± 149	705 ± 128	1,019 ± 142	1,541 ± 172	3,507 ± 126	<0.001
Apgar 1 min <7, n (%) †	5 (50)	4 (57)	3 (43)	6 (67)	6 (55)	0 (0)	0.199
Apgar 5 min <7, n (%) †	1 (10)	2 (29)	0 (0)	4 (44)	1 (9)	0 (0)	0.095
Cesarean delivery, n (%) †	8 (80)	6 (86)	7 (100)	3 (33)	4 (36)	6 (100)	0.003

* Data presented as mean ± SEM and analyzed by 1-way ANOVA;

† Data presented as n (%) and analyzed by Chi square tests;

‡ Data presented as median [interquartile range] and analyzed by Kruskal-Wallis ANOVA;

§ Data presented as n (%) and Fisher's exact tests.

PPROM: preterm premature rupture of the membranes