CD74-Downregulation of Placental Macrophage-Trophoblastic Interactions in Preeclampsia

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Rationale: We hypothesized that cluster of differentiation 74 (CD74) downregulation on placental macrophages, leading to altered macrophage-trophoblast interaction, is involved in preeclampsia.

Objective: Preeclamptic pregnancies feature hypertension, proteinuria, and placental anomalies. Feto-placental macrophages regulate villous trophoblast differentiation during placental development. Disturbance of this well-balanced regulation can lead to pathological pregnancies.

Methods and Results: We performed whole-genome expression analysis of placental tissue. CD74 was one of the most downregulated genes in placentas from preeclamptic women. By reverse transcriptase-polymerase chain reaction, we confirmed this finding in early-onset (<34 gestational week, n=26) and late-onset (≥34 gestational week, n=24) samples from preeclamptic women, compared with healthy pregnant controls (n=28). CD74 protein levels were analyzed by Western blot and flow cytometry. We identified placental macrophages to express CD74 by immunofluorescence, flow cytometry, and RT-PCR. CD74-positive macrophages were significantly reduced in preeclamptic placentas compared with controls. CD74-silenced macrophages showed that the adhesion molecules ALCAM, ICAM4, and Syndecan-2, as well as macrophage adhesion to trophoblasts were diminished. Naive and activated macrophages lacking CD74 showed a shift toward a proinflammatory signature with an increased secretion of tumor necrosis factor-alpha, chemokine (C-C motif) ligand 5, and monocyte chemotactic protein-1, when cocultured with trophoblasts compared with control macrophages. Trophoblasts stimulated by these factors express more CYP2J2, sFlt1, TNFa, and IL-8. CD74-knockout mice showed disturbed placental morphology, reduced junctional zone, smaller placentas, and impaired spiral artery remodeling with fetal growth restriction.

Conclusions: CD74 downregulation in placental macrophages is present in preeclampsia. CD74 downregulation leads to altered macrophage activation toward a proinflammatory signature and a disturbed crosstalk with trophoblasts. (Circ Res. 2016;119:55-68. DOI: 10.1161/CIRCRESAHA.116.308304.)

Key Words: immunology | macrophages | preeclampsia | pregnancy | trophoblasts

Preeclampsia is a pregnancy-related disorder characterized by a new-onset hypertension (>140/90 mmHg) after the 20th week of pregnancy and proteinuria (>300 mg/L per 24-hour) or in association with thrombocytopenia, impaired liver function, development of renal insufficiency, pulmonary edema, or new-onset cerebral or visual disturbances. Overall, 5% to 10% of all pregnancies worldwide develop preeclampsia, the leading cause of morbidity and mortality in mother and child.

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Immune cell and trophoblastic–cell interactions facilitate placental growth by a well-balanced regulation, including interactions between maternal immune cells and extravillous trophoblast as well as between fetoplacental immune cells and villous trophoblast. Pregnancy initiates a profound adaptation of the maternal immune system to the semiallogeneic fetus, and a significant inflammatory response has been described in preeclamptic women. Chemokines such as monocyte chemotactic protein-1 (MCP-1) and chemokine (C–C motif) ligand 5 (CCL5) are elevated in preeclampsia and enhance trophoblast apoptosis in vitro.9,10 Moreover, invasion, migration, and proliferation of extravillous trophoblasts seem to be regulated by local cytokine concentrations.11–14

Hofbauer cells are placent al macrophages of fetal origin and play a direct role in early placent al development. They differentiate from progenitor cells within the population of mesenchymal cells in the villous stroma or from penetration of embryonic/fetal bone marrow–derived (BMD) monocytes into the villous stroma.15 Hofbauer cells are associated with several pregnancy complications, such as chorioamnionitis, spontaneous abortion, and fetal metabolic storage disease.16 Hofbauer cells contribute to the placental expression of antiangiogenic factors and their dysregulation in preeclamptic placenta.17

The human leukocyte antigen (HLA) class II histocompatibility antigen-γ chain, also known as cluster of differentiation 74 (CD74) when expressed on cell surfaces, is the major histocompatibility complex (MHC) II invariant chain (II) protein that is involved in antigen presentation and crucial for biogenesis.18 CD74 is also a high-affinity binding protein for the pleiotropic inflammatory cytokine macrophage migration inhibitory factor (MIF). After its activation on the cell membrane, CD74 transduces signaling and triggers various downstream responses, including inflammatory protein expression and modulation of cell survival.19 MIF also acts as a major regulator of inflammatory cell recruitment and atherogenesis by activating CXC chemokine receptor2/4.20 In the first-trimester placenta, trophoblasts express and release MIF that modulates monocyte activity.21–23 Furthermore, CD74 has been described in a soluble form (sCD74) that neutralizes MIF signal transduction in patients with autoimmune liver disease.24 Soluble CD74 plasma levels correlate with the concentration of a complex that is formed between sCD74 and soluble HLA-DR (sCD74/sHLA-DR).25 Interestingly, sHLA-DR levels are significantly lower in the circulation of preeclamptic women when compared with women with normally progressing pregnancies.26 On the basis of microarray gene expression data from clinical specimens, we tested the hypothesis that preeclampsia is associated with a CD74-induced disturbed placentation. We used in vitro models and a knockout mouse model in vivo to study the pathophysiological significance of CD74.

Methods

Patients

Human placenta, decidua, and blood sampling were approved by the Regional Committee of Medical Research Ethics in Eastern Norway and the Medical Faculty of Charité Berlin. Placent al biopsies and decidu al tissue were obtained from 50 preeclamptic women and 28 women with normotensive and uncomplicated pregnancies. The preeclampsia group was divided into early-onset preeclampsia (<34 gestational week, n=26) and late-onset preeclampsia (≥34 gestational week, n=24). Patient characteristics are shown in Online Table I.

Animals

Local authorities (LaGeSo, Berlin, Germany) approved all experiments. Primary cultures of macrophages were generated from the bone marrow of male 10 to 12-week-old wild-type (WT; C57Bl/6JolaHsd) mice (Harlan Laboratories, Rossdorf, Germany) or CD74-knockout (B6-Cd74tm1) mice. To evaluate the preeclampsia phenotype, WT (C57Bl/6JolaHsd) and CD74-knockout (B6-Cd74tm1) mice were bred. Doppler studies were performed on days 15 and 17 on anesthetized (1.5% isoflurane) mice. The hair was removed from the abdomen, and prewarmed gel was used as an ultrasound-coupling medium. The pregnant mice were imaged with an ultrasound biomicroscope and a 30-MHz or 40-MHz transducer at 30 frames/s (Model Vevo 660, VisualSonics Inc). Peak systolic velocity (PSV) and end-diastolic velocity (EDV) were measured and the resistance index (RI=PSV–EDV/PSV) was calculated.

Isolation of Primary Cells From Human Placenta

All placentas were removed from fetal membranes, basal plate, umbilical cord, and fibrotic tissue. Villous tissue was minced and washed several times. Various trypsin (Sigma) and DNase I (Roche) digestion steps were used and supernatants were filtered and collected in New Born Calf Serum (Biochro me). For Hofbauer cell isolation supernatant was discarded, and undigested tissue was digested further with collagenase A (Roche), DNase I and cell suspensions were loaded on Percoll (GE Healthcare) gradual gradients. For primary

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ALCAM</td>
<td>activated leukocyte adhesion molecule</td>
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<tr>
<td>BMD</td>
<td>bone marrow derived</td>
</tr>
<tr>
<td>CCL5</td>
<td>chemokine (C–C motif) ligand 5</td>
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<tr>
<td>CD74</td>
<td>cluster of differentiation 74</td>
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<tr>
<td>CK7</td>
<td>cytokeratin 7</td>
</tr>
<tr>
<td>CYP2J2</td>
<td>cytochrome P450 subfamily 2J polypeptide 2</td>
</tr>
<tr>
<td>EDV</td>
<td>end-diastolic velocity</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>ICAM4</td>
<td>intracellular adhesion molecule 4</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>MAPK3</td>
<td>mitogen-associated protein kinase 3</td>
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<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
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<tr>
<td>MIF</td>
<td>macrophage migration inhibitory factor</td>
</tr>
<tr>
<td>PKD1</td>
<td>polycystic kidney disease gene 1</td>
</tr>
<tr>
<td>PLGF</td>
<td>placental growth factor</td>
</tr>
<tr>
<td>PSV</td>
<td>peak systolic velocity</td>
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<tr>
<td>RHOA</td>
<td>Ras homolog gene family, member A</td>
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<tr>
<td>RI</td>
<td>resistance index</td>
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<tr>
<td>sCD74</td>
<td>soluble cluster of differentiation 74</td>
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<tr>
<td>SDC2</td>
<td>syndecan-2</td>
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<tr>
<td>sFlt1</td>
<td>soluble Fms-like tyrosine kinase 1</td>
</tr>
<tr>
<td>SGHPL-4</td>
<td>primary human first trimester extravillous trophoblasts</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
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<td>WT</td>
<td>wild-type</td>
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RI=(PSV−EDV)/PSV was calculated.

MIF also acts as a major regulator of inflammatory cell recruitment and atherogenesis by activating CXC chemokine receptor2/4. In the first-trimester placenta, trophoblasts express and release MIF that modulates monocyte activity. Furthermore, CD74 has been described in a soluble form (sCD74) that neutralizes MIF signal transduction in patients with autoimmune liver disease. Soluble CD74 plasma levels correlate with the concentration of a complex that is formed between sCD74 and soluble HLA-DR (sCD74/sHLA-DR). Interestingly, sHLA-DR levels are significantly lower in the circulation of preeclamptic women when compared with women with normally progressing pregnancies. On the basis of microarray gene expression data from clinical specimens, we tested the hypothesis that preeclampsia is associated with a CD74-induced disturbed placentation. We used in vitro models and a knockout mouse model in vivo to study the pathophysiological significance of CD74.

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trophoblasts, cells were negatively immunopurified using HLA-ABC (DakoCytomation) and magnetic dynabeads (Dynal Biotech).

**Macrophage Preparation and Cell Culture**

Macrophages were generated from 2 different origins: human peripheral blood mononuclear cells and mouse BMD. Peripheral blood mononuclear cells–derived macrophages: 80 mL of blood were drawn from human healthy donors. The study was approved by the Regional Committee of Medical Research Ethics (Charité). Peripheral blood mononuclear cells were purified by Ficoll gradient centrifugation and an adhesion step.

BMD macrophages: Cells were isolated from the femur and tibia of freshly euthanized mice. For macrophage differentiation, 10×10⁶ BMD cells were cultivated in 50 mL of differentiation media for 7 days in sealed, hydrophobic Teflon bags (FT FEP 100 C [DuPont], American Durafilm).

SGHPL-4 cells derived from primary human first-trimester extravillous trophoblasts (transfected with the early region of SV40) were a kind gift from Judith E. Cartwright (St George’s University of London, London, United Kingdom).

**Microarray Analysis**

Microarray analysis performed on placental tissue showed downregulation of CD74 expression by 3 different nucleotide sequences (ILMN_1761464, ILMN_2379644, and ILMN_1736567). This observation was confirmed in a different cohort of patients, dividing the preeclamptic group into early onset (<34 gestational week) and late onset (>34 gestational week) of preeclampsia (Online Table I) by real-time reverse transcriptase-polymerase chain reaction. We found that CD74 was significantly downregulated in the early-onset preeclampsia by a factor of 2.3 (P<0.001) and the late-onset preeclampsia by a factor of 1.9 (P<0.01; Figure 1A). Western blot analysis of 4 controls versus 4 preeclamptic placentas confirmed this finding. CD74 protein level was clearly diminished in both early- and late-onset preeclamptic placentas (Figure 1B). Moreover, flow cytometry analysis on the whole placental cell population of 6 healthy and 10 late-onset preeclamptic placentas showed a significantly lower presentation of CD74 in cells of late-onset preeclamptic placentas (Figure 1C). Decidua showed no differential CD74 expression between groups (Online Figure IA). In the preeclampsia group, placental CD74 expression negatively correlated to the serum ratio of the soluble Fms-like tyrosine kinase 1 (sFlt1) and the placental growth factor (PLGF), the sFlt1/PLGF ratio, but not in controls (Online Figure ID). CD74 not only serves as invariant chain for MHC class II proteins and as an MIF receptor on the cell surface but also circulates as a soluble ectodomain, termed sCD74. We thus also analyzed serum sCD74 levels in our cohort and detected somewhat lower mean levels in both the preeclamptic groups compared with controls, although these results were not statistically significant (Online Figure IB). Circulating levels of the binding partner of sCD74, MIF, were correspondingly enhanced during preeclamptic disease, but without reaching statistical significance (Online Figure IC).

**Localization of CD74 in Placental Macrophages (Hofbauer Cells)**

Following differential expression of CD74 in preeclamptic placentas, we identified the specific cell type that expresses CD74. Given the low amounts of lymphocytes in placenta, we focused on CD74 levels in 2 main placental populations, trophoblasts (cytokeratin 7 [CK7]+) and macrophages (CD14+). It seemed that in healthy control placentas only 2.6±0.8% of trophoblasts were positive for CD74 (CK7+CD74+), in contrast to 93.1±1.1% of macrophages (CD14+CD74+; Figure 2A). Furthermore, immunofluorescence costaining was performed on placental villous tissue for trophoblasts (CK7) and Hofbauer cells (CD163) with CD74. We could confirm that trophoblasts were negative for CD74 (CK7+CD74−), whereas Hofbauer cells were positive (CD163+CD74+; Figure 2B).

To investigate CD74 mRNA expression levels, we isolated primary trophoblasts and primary Hofbauer cells from healthy human term placentas and compared them with placental tissue, early trophoblast cell line (SGHPL-4) and blood-derived macrophages (human M(IL-4)). We confirmed that isolated Hofbauer cells showed high levels of CD74 expression, similar to human M(IL-4) and M(−), whereas primary trophoblasts and SGHPL-4 cells showed low levels (Online Figure II). Accordingly, in Hofbauer cells and blood-derived macrophages, but not in SGHPL-4 cells, the phosphorylation of ERK (p44/42) could be activated by stimulation with the CD74 ligand MIF (Online Figure III).

To combine both the findings, the observed placental downregulation of CD74 in preeclamptic placentas and the expression of CD74 on placental macrophages, we then compared CD74 levels expressed on CD14+ Hofbauer cells from healthy and preeclamptic placentas. Significantly, fewer plental CD14+ macrophages coexpressed CD74 in late preeclampsia (81.2±4.4%), compared with healthy controls (93.1±1.1%; P<0.05; Figure 2C, left). In addition, CD74+ cells of preeclamptic placentas showed lower intensity of CD74 staining when compared with controls, shown by mean fluorescence intensity of CD74 (16283±1244 versus 7693±1392 AU, P<0.01; Figure 2C, middle). The frequency of CD14+ macrophages did not differ between control and preeclamptic placentas (Figure 2C, right).

**Impaired Function of Macrophages Lacking CD74**

Since Hofbauer cells (Figure 3) are considered to be M2 (also named alternatively activated) macrophages, we aimed to establish an in vitro preeclamptic model of CD74-downregulated Hofbauer cells by transfecting M(IL-4) with small-interfering RNA (siRNA) against CD74 (siCD74) and an appropriate nontargeting control (Figure 4). However, the current characterization of placental macrophages might not be sufficient about activation status, we therefore used M(−) for silencing protocols additionally. Downregulation of CD74 was confirmed by gene expression via real-time reverse transcriptase-polymerase chain reaction (Online Figure IVA) and at the protein level by Western blot (Online Figure IVB; data...
shown for M(IL-4)). mRNA was processed, and differential expression (nontargeting versus siCD74; n=6 each) was revealed by whole transcriptome analysis. Subdued parametric 3-way ANOVA resulting $P$ values were Benjamini Hochberg false discovery rate corrected; furthermore, probes undergoing 5% false discovery rate in silencing were average linkage-clustered using standardized signal values and the Euclidean distance function. Thirty probes, representing 27 known genes, were significantly differentially expressed by CD74 silencing (5% false discovery rate), 25 of them were downregulated in CD74-silenced samples and 2 were upregulated and represented on the heat map (Figure 4A, left). These 27 genes were matched to the literature in respect to placenta. We found that Ras homolog gene family, member A (RHOA), mitogen-associated protein kinase 3 (MAPK3), and polycystic kidney disease gene 1 (PKD1) were linked to pregnancy and are highlighted in orange. For further analysis, genes that followed false discovery rate <65% were plotted and are shown in Figure 4A (right). To determine possible interactions of differently regulated genes, data sets of these 226 genes with altered expression were analyzed using ingenuity pathway analysis tool. The top 10 list of canonical pathways was identified by ingenuity pathway analysis. The proinflammatory IL-1–signaling pathway was the most significantly enriched. Among the identified biological functions, we found cell morphology, connective tissue disorders, and immunologic diseases (Online Figure V). Strongly downregulated genes within the 226 dysregulated genes were adhesion molecules—activated

Figure 1. Cluster of differentiation 74 (CD74) is downregulated in preeclamptic placenta. A, CD74 expression is downregulated in placentas of preeclamptic (PE) women compared with healthy women (control; n=28). PE is subdivided in early-onset PE (delivery <34 wk of gestation; gray bar; n=28) and late-onset PE (delivery ≥34 wk of gestation; black bar; n=24; **$P$<0.01; ***$P$<0.001; Bonferroni multiple comparisons test). B, CD74 protein (33 and 35 kDa) is lowered in placenta lysates of early- and late-onset PE (n=2 each). C, Confirmation by flow cytometry; representative CD74-positive gating is shown for whole placenta cell population (left). Mean fluorescent intensity of CD74 is lowered in general placenta cell population of late PE vs control (right; n=10 [PE] and 6 [control]; ***$P$=0.001; Mann–Whitney test).
Figure 2. Cluster of differentiation 74 (CD74) is highly expressed in placental macrophages (Hofbauer cells) and downregulated in preeclamptic Hofbauer cells. A, By flow cytometry; representative cytokeratin 7 (CK7)–positive gating (trophoblast marker) and CD14–positive gating (macrophage marker) are shown for whole placenta cell population (left) of healthy controls. Solely 2.6±0.8% of (Continued)
leukocyte cell adhesion molecule (ALCAM), intracellular adhesion molecule 4 (ICAM4), and syndecan-2 (SDC2)—which are presented in red (Figure 4A, right). We confirmed ALCAM, ICAM4, and SDC2 downregulation in M(IL-4) lacking CD74 by real-time reverse transcriptase-polymerase chain reaction (n=27 each; Online Figure VI).

Because gene expression of adhesion molecules was downregulated in macrophages lacking CD74, adhesion assay with M(IL-4) macrophages and first-trimester trophoblast cell line SGHPL-4 was performed. There was significantly less M(IL-4) adherence to the SGHPL-4 layer in the siCD74-treated group when compared with nontargeting, as documented by microscopy and measured by fluorescence intensity (Figure 4B).

To reveal the impact of CD74 deficiency on M2 macrophages, we also generated BMD macrophages from CD74−/− mice. BMD M(−) and M(IL-4+IL-13) showed high CD74 expression when derived from WT, which was absent in CD74−/− macrophages (Online Figure VII). First, we activated CD74−/− and WT macrophages with IL-4+IL-13 to promote a M2 phenotype and analyzed the secretion of different cytokines (Figure 5A). Surprisingly, M2 macrophages from CD74−/− mice secreted significantly more TNF-α (1.8-fold) and CCL5 (1.7-fold) compared with WT-derived macrophages, indicating a shift toward a proinflammatory phenotype. Coculturing M2 CD74−/− macrophages with trophoblast cell line SGHPL-4 further increased the secretion of these cytokines TNF-α (2.7-fold) and CCL5 (2.7-fold). Interestingly, the secretion of chemokine MCP-1 was 4.1-fold only induced in BMD M(IL-4+IL-13) from CD74−/− (compared with WT) when cocultured with SGHPL-4. SGHPL-4 cell count was significantly lowered when cocultured with BMD M(IL-4+IL-13) from CD74−/− compared with BMD M(IL-4+IL-13) from WT (4284±225 versus 5200±252 cells; Figure 5B). SGHPL-4 cells stimulated with the combination of MCP-1, CCL5, and TNF-α for 6 and 24 hours expressed more cytochrome P450 subfamily 2J polypeptide 2 (CYP2J2), sFlt1, TNFα, and IL-8. The sFlt1/PLGF ratio was also enhanced (Figure 5C).

To reveal the impact of CD74−/− on M1 and M2 macrophage activation, we analyzed for M1 activation the proinflammatory marker genes Tnfα, Il-6, Ccl5, nitric oxide synthase 2 (Nos2), and Mcp-1 and for M2 activation resistin-like molecule α1 (Fizz1), mannose receptor 1 (Mrc-1), and programmed death ligand 2 (Pd-l2) in BMD M(−) and M(IL-4+IL-13) from CD74−/− compared with WT (Figure 6). Arginase 1 (Arg1) is known to be activated after M1 and M2 stimuli. Lipopolysaccharides (LPS) used to induce M1 activation (M[LPS]). The activation by IL-4+IL-13 and LPS led to a typical marker gene signature in BMD macrophages from both origins, CD74−/− and WT. Nonactivated M(−) macrophages from CD74−/− origin showed an enhanced expression of Tnfα (1.6-fold), Il-6 (2.9-fold), Ccl5 (3.7-fold), Nos2 (14-fold), and Mcp-1 (1.5-fold) compared with M(−) WT...
macrophages, indicating a shift toward a proinflammatory signature. Under M2 conditions, Tnfa and Il-6 were also higher expressed in M(IL-4+IL-13) CD74−/− macrophages. LPS activation (M[LPS]) led to a higher expression of Tnfa, Il-6, Nos2, and Mcp-1 in CD74−/− macrophages, indicating again a shift toward a proinflammatory macrophage phenotype.

Disturbed Placental/Fetal Phenotype of CD74-Knockout (CD74−/−) Mice

To test the hypothesis that CD74 is important for pregnancy and fetal development, we analyzed the pregnancy and the uteroplacental unit of the CD74−/− mice. CD74 expression was absent on protein level in the placenta of CD74−/− and clearly detectable in WT mice (Online Figure VIII). Fetal weight on day 18 was significantly lower in CD74−/− mice (0.71±0.02 g) compared with WT mice (0.91±0.03 g; Figure 7A). Placenta weight (0.1 g in CD74−/− versus 0.11 g in WT) and litter size (7 in CD74−/− versus 9 in WT pups) were also diminished. Histomorphological analysis revealed a significant overall reduction of the placental area in CD74−/− mice, compared with WT placentas (Figure 7B). In addition, CD74−/− placentas presented significant structural changes, as the ratio between the 2 functional placental zones, the labyrinth and the junctional zones, was increased in placentas from CD74−/− mice compared with WT controls (Figure 7B, bottom). This increase was because of a significant reduction of the junctional zone in CD74−/− placentas, whereas the labyrinth area remained unchanged between CD74−/− and WT mice (Online Figure IX). The spiral artery remodeling was also impaired in arteries of the decidua basalis and mesometrial tissue (Figure 7C and 7D). Areas are indicated in Online Figure XA. CD74−/− mice showed significantly more α-actin, representing an abnormal remodeling process (Figure 7C and 7D). Arteries of CD74−/− mice were smaller, indicated by an artery area (µm²) and a perimeter (µm)
when compared with WT mice (Online Figure XB). Doppler waveform analysis of the distal uterine artery showed a significant increase of the RI of the CD74−/− mice (0.51±0.02 RI) compared with WT mice (0.44±0.02 RI; Online Figure XI).

A putative maternal syndrome was characterized by blood pressure and albuminuria (Online Figure XII). However, on day 15 of pregnancy, mean arterial pressure and albuminuria were not significantly changed in CD74−/− mice. To analyze the effects of whole body knockout (CD74−/−) in the female mice on fetal growth restriction and lowered placental weights, we transferred fertilized oocytes from CD74−/− mice to WT foster mothers. Fetuses and placentas resulting from this model were also smaller compared with fetuses and placentas resulting from WT fertilized oocytes (Online Figure XIII).

**Discussion**

We present novel data to support the notion that CD74 is significantly downregulated in third-trimester preeclamptic placentas. We successfully localized the origin of CD74 expression to placental macrophages (Hofbauer cells) and inversely correlated...
the placental CD74 expression to the serum sFlt1/PLGF ratio that is enhanced in the preeclamptic circulation. In maternal blood, the shed-soluble CD74 and its ligand MIF were not statistically significantly altered in the circulation of preeclamptic women. Importantly, macrophages that lack CD74 showed a dysregulated interaction with trophoblasts. The adhesion of such macrophages to trophoblasts was diminished, whereas CD74-deficient macrophages exhibited a proinflammatory phenotype when cocultured with trophoblasts. Trophoblasts activated by those proinflammatory stimulus expressed more vasoactive and antiangiogenic factors. M1 and M2 activation under LPS and IL-4+IL-13 led to the induction of the respective signature genes. Interestingly, M(IL-4+IL-13) CD74−/− M2 macrophages also expressed proinflammatory genes, suggesting a shift toward an altered M2 phenotype. Importantly, CD74−/− mice showed altered placenta morphology, evidenced by a smaller junctional zone and an impaired spiral artery remodeling. This abnormal placentation was accompanied by fetal growth restriction and an increased RI of the distal uterine arteries. Our results suggest a novel link between preeclampsia and the regulatory interaction of placental macrophages and villous trophoblasts with a specific role for CD74.

The most studied function of CD74 is its chaperone activity, in which CD74 acts as an invariant chain for MHC class II proteins and is crucial for antigen presentation. Because of the importance of antigen presentation in the recognition of fetal cells by the mother, it is surprising that little is known about the expression of CD74 in the placenta. Peyman et al showed that trophoblasts do not present MHC class II proteins and CD74 on their cell surface. Moreover, those genes seem to be inactive because the mRNA expression was not inducible by interferon-γ, which has been shown to upregulate

Figure 6. Proinflammatory signature of macrophages derived from cluster of differentiation 74 (CD74)–knockout mice (CD74−/−). In vitro differentiated bone marrow–derived (BMD) macrophages (M(−)) from CD74−/− mice express more tumor necrosis factor-α (Tnfα), interleukin 6 (Il-6), chemokine (C–C motif) ligand 5 (Ccl5), nitric oxide synthase 2 (Nos2), and monocyte chemoattractant protein-1 (Mcp-1) compared with BMD M(−) from wild-type (WT) mice. Tnfα and Il-6 were also higher expressed in BMD M(IL-4+IL-13) from CD74−/− mice compared with WT. Marker for lipopolysaccharide (LPS) signature (Nos2, Ccl5, Il-6, Tnfα, and Mcp-1) and IL-4+IL-13 signature (arginase 1 [Arg1], resistin-like molecule α-1 [Fizz1], mannose receptor-1 [Mrc-1], and programmed death ligand 2 [Pd-l2]) were fully activated in M(LPS) and M(IL-4+IL-13), respectively, and proinflammatory activation by LPS was further increased by CD74−/− in regard of Tnfα, Il-6, Nos2, and Mcp-1 (n=6 each; *P<0.05, **P<0.01, ***P<0.001; unpaired t-test).
Figure 7. Fetal growth restriction, disturbed placenta morphology and impaired spiral artery remodeling in cluster of differentiation 74 (CD74)-knockout mice (CD74−/−). A, Pup and placental weights were lower in CD74−/− (n=32) vs wild-type (WT, n=33). Litter size was also decreased in CD74−/− (n=17) vs WT (n=28; *P<0.05, **P<0.01 and ****P<0.0001; Mann–Whitney test). B, Representative pictures of Masson-stained midsagittal placental tissue sections used to carry out the histomorphological analysis (top). (Continued)
these proteins in macrophages. These observations are in line with our findings. We also found that CD74 is expressed by placental macrophages and not by trophoblast cells. In human placent al explants infected by Toxoplasma gondii, a model for induction and suppression of infection, CD74 was detected in first but not in third-trimester trophoblasts, as well as in Hofbauer cells.30 However, colocalization of CD74 with a marker-like CD163 for macrophages or CK7 for trophoblast was not performed. The expression of CD74 in general was higher in first-trimester explants. Interestingly, in this study the production and secretion of the CD74 ligand MIF was enhanced in first-trimester placenta after the activation of inflammatory processes by pathogens. MIF may play an essential role as an autocrine/paracrine mediator in placental infection.23,30 In this context, we found that expression of CD74 is downregulated in preeclampsia suggests a disturbance of the normally balanced immune response to antigens in the pre eclamptic placenta. MIF expression and concentrations were described in pregnancy and preeclampsia by Cardaropoli et al.31 Only in serum of preeclamptic women accompanied by fetal growth restriction they found significantly higher MIF levels compared with controls. However, that study also failed to show a statistically significant upregulation in the pre eclamptic group without fetal growth restriction, which is in line with our findings.

Inflammatory dysfunction in preeclampsia is mostly described on the maternal side of the uteroplacental unit or in maternal circulation. Brewster et al.32 described that the monocyte secretory capacity for different cytokines, including MCP-1 and TNF-α, is higher in preeclamptic women than in controls. MCP-1 secretion can be stimulated by TNF-α and IL-1β in first trimester–derived decidual explants.33 The chemokines MCP-1 and CCL5, as well as the adhesion molecules ICAM-1 and VCAM-1 (vascular cell adhesion molecule), are elevated in preeclampsia compared with healthy pregnancies, resulting in an overall proinflammatory systemic environment.5,7 Circulating levels of the inflammatory cytokines TNF-α, IL-10, and interferon-γ are controversially discussed.34 Our results from the in vitro model also suggest an upregulated expression of macrophage signaling molecules such as MCP-1, TNF-α, and CCL5 as well as a diminished adhesion of macrophages to trophoblasts in preeclampsia that may be caused by downregulated CD74. The in vitro findings show a dysregulation of the well-balanced interaction between the villous trophoblasts and the placental macrophages toward a proinflammatory phenotype. However, this does not illuminate the nature of the differences in circulatory in the maternal system. Fest et al.35 could show that the human THP-1 monocyte line expressed and secreted more cytokines and chemokines when cocultured with trophoblasts compared with mononuclear culture. Among these factors were MCP-1 and CCL5. Our findings are in line with this observation. We also show an effect of trophoblasts on macrophages about the secretion of TNF-α, MCP-1, and CCL5. Furthermore, we showed that this effect is enhanced by CD74 downregulation. Further induction of an already existing robust inflammatory response during pregnancy by inflammatory cytokines and chemokines is already described in preeclampsia.3,4 Here, we could show that the activation of a trophoblast cell line by TNF-α, MCP-1, and CCL5 led to an upregulation of the vasoactive CYP2J2, the antiangiogenic factor sFlt1 and the proinflammatory TNFα and IL-8. All these factors are described to be mechanistically involved in the preeclamptic syndrome.27,34,35,36 Recently, we described the fact that the CYP2J2 expression is upregulated in preeclamptic trophoblasts.37 This uteroplacental upregulation resulted in enhanced CYP2J2 metabolite levels in the circulation of preeclamptic women. Furthermore, we tested the vasoactive function the metabolite and could show that it induced a chronotropic effect, an endothelial dysfunction and a downregulation of the KCa1.1 channel activity. Its function was synergistic to angiotensin II. In preeclampsia, angiotensin II sensitivity, endothelial dysfunction, and alterations in endothelium-dependent vascular contractile properties are part of the maternal syndrome. We now can conclude that the recently described CYP2J2 upregulation in preeclamptic trophoblasts could be a mechanism of the here described CD74 dependent macrophage–trophoblast interaction. We also could show that CD74−/− macrophages show a proinflammatory shift toward an M(LPS) (or classical macrophage [M1]) phenotype. This result is in line with recent findings. Similar shift toward proinflammatory M1 phenotype was also described in preeclamptic placentas were the increase of M1 macrophages and the decrease of M2 macrophages lead to an acute atherosis.38

Hofbauer cells may play a direct role in early placental development because early placental vasculogenesis is influenced by close contact of Hofbauer cells with endothelial progenitor cells in primitive vessels in the first-trimester placenta.39 With respect to the importance of Hofbauer cells in the early placenta, our study of early third-trimester placentas show the relevance of CD74 and Hofbauer cells in the middle stage of pregnancy, which may be of importance in the pathophysiology of preeclampsia and fetal growth restriction. We can only speculate that CD74 downregulation is already a feature of immunologic dysregulation at the beginning of pregnancy or even before conception, the latter being viewed as relevant for the development of preeclampsia or fetal growth restriction.40 Little is known about the transcriptional regulation of CD74. CD74 expression is described to be increased in tissue injury disorders and decreased in brain tissue in schizophrenia and bipolar disorder.41,42 However, none of these studies could reveal the regulatory mechanisms that underline the regulation of CD74 expression. The regulation of CD74 in different cell types correlates with glucose concentrations and an immune activation.41,43 The inaccessibility of tissue from histiotropic

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**Figure 7 Continued.** Zones are marked and indicated by L (labyrinth zone) and Jz (junctinal zone). The overall size of the placenta was decreased in CD74−/− tissues (bottom), whereas the ratio between the labyrinth/junctinal zones was enhanced in CD74−/− (n=13) in comparison with WT (n=17). The area of the junctional zone of CD74−/− placentas was smaller compared with those of WT (**P<0.01, ***P<0.001; unpaired t test). C, α-actin staining is shown in representative pictures of arteries. Arrows are indicating α-actin–stained C. bar of 100 μm. Arrows are indicating α-actin–stained C. bar of 100 μm. **Figure 7 Continued.** Zones are marked and indicated by L (labyrinth zone) and Jz (junctinal zone). The overall size of the placenta was decreased in CD74−/− tissues (bottom), whereas the ratio between the labyrinth/junctinal zones was enhanced in CD74−/− (n=13) in comparison with WT (n=17). The area of the junctional zone of CD74−/− placentas was smaller compared with those of WT (**P<0.01, ***P<0.001; unpaired t test). C, α-actin staining is shown in representative pictures of arteries. Arrows are indicating α-actin–stained C. bar of 100 μm. Arrows are indicating α-actin–stained C. bar of 100 μm.
placentas let us just speculate about the mechanisms that lead to the downregulation of CD74 in the preeclamptic placental macrophages. Glucose regulates the heme oxygenase-1 that is a central player for immune regulation.48 Furthermore, glucose regulates the exosomal signaling by placental cells and the release of cytokines.49 It might be that a dysregulation in glucose metabolism at first trimester in pregnancies has adverse outcome on the CD74 expression and, therefore, associates with placental insufficiency. Currently, no single-nucleotide polymorphism is described for the CD74 gene or its promoter region. Analysis of single-nucleotide polymorphisms and their association to the genesis of the preeclamptic syndrome are growing. Association studies of polymorphisms in inflammatory genes involved in preeclampsia are controversial.46 However, further studies to reveal the mechanisms that underlie the transcriptional regulation of CD74 are important.

The CD74−/− mouse model was established and first described in 1993 by Bikoff et al.47 Most publications described the role of CD74 in regulating MHC class II expression and function.48 Recently, CD74−/− mice were described to develop spontaneous emphysema in association with MIF.49 Mun et al50 isolated bone marrow cells from CD74−/− mice and described the mice as indistinguishable from WT littermates in their general health, growth rate, as well as breeding performance. However, this does not reflect our observations. This result could be because of different murine backgrounds because the previous authors crossed heterozygous CD74-knockout mice to generate homozygous CD74−/− littermates or to an insufficient characterization of fetuses and placentas. Herein, we report that CD74 deficiency was associated with an altered placentalization at late gestation, evidenced by smaller placentas and an impaired spiral artery remodeling. Placentas from CD74−/− mice also revealed an increased LJZ ratio, which was mainly because of a decrease of the junctional zone area. The junctional zone is composed of spongiotrophoblast, glycogen cells, and a layer of giant cells reaching the decidual tissue.51 Although the function of the junctional zone in mice is not yet fully understood, it has been shown to support the growth and expansion of the labyrinth.52 Moreover, the junctional zone synthesizes and secretes a vast array of cytokines and hormones such as placental lactogens, hereby maintaining fetal growth.53–54 Hence, the decreased junctional zone observed in CD74−/− mice at late gestation and the impaired spiral artery remodeling could account for the increased RI of the distal uterine arteries and the decreased fetal weight. Moreover, we provide evidence that although the area of the labyrinth is unaffected in CD74−/− mice, the relative fraction of the placenta occupied by the labyrinth is increased. This relative increase in the size of the labyrinth has been reported to naturally occur in small placentas and can be interpreted as an adaptation attempt to meet the demands for fetal growth.55 The labyrinth comprises a complex vascular network of maternal and fetal blood vessels in close proximity, hereby promoting nutrient and gas transfer from mother to fetus. Interestingly, it was shown that the relative increase in the labyrinth from small placentas occurs at the expense of the junctional zone, which could provide an explanation for the decrease in the area of the junctional zone observed in the CD74−/− placentas. We here assessed placental tissue from CD74−/− mice and respective WT controls late in gestation and hence, can only speculate that the adaptive changes observed in the placentas of CD74−/− mice may have occurred earlier in gestation. A limitation of the mouse model might be the knockout effect on other cell types than the placental macrophages and the whole body knockout effects in the maternal body. To exclude the CD74−/− effect in organs of the female mice, we also provided the CD74−/− 2-cell stage embryo transfer in WT mice. The observed fetal and placental growth restrictions underline the significance of the CD74−/− on the utero-placental unit. However, in this model, we just can speculate that the effects are macrophage driven and not costimulated by CD74−/− in other placental cell types. Further experiments with a placental macrophage–specific knockout model could completely rule out the interaction between macrophages and trophoblasts. The maternal syndrome was not developed in CD74−/− mice. One explanation for the lack of hypertension in CD74−/− mice could be related to the C57BL/6 genetic background of the knockout mice. European C57BL/6 are often resistant to hypertension. Therefore, we cannot completely rule out a role of CD74 on blood pressure. Our hypothesis is that the CD74 downregulation leads to an adverse outcome in placentaion, resulting in vulnerability for preeclampsia and intrauterine growth restriction. We hypothesize that additional triggers could promote a maternal syndrome, and further studies will be necessary to investigate which second hit is necessary in addition to downregulation of CD74 to promote the full clinical picture of preeclampsia. Furthermore, our findings are in line with the clinical discussion of preeclampsia and intrauterine growth restriction. Both pregnancy complications share risk factors, molecular pathways and the risk for developing future cardiovascular.56 Moreover, as preeclampsia is a complex syndrome, the lack of CD74 in the mouse model we here used may have been compensated by other pathways. Such redundancy of single pathways in the context of maintaining successful reproduction has been described in a number of genetically engineered mice.57

CD74 is significantly downregulated in third-trimester preeclamptic placentas. Therefore, we propose that CD74 might be involved in the development of preeclampsia. Our data suggest that CD74 is a potential candidate for controlling the well-balanced regulation between the villous trophoblasts and the placental macrophages. Thus, studies on CD74 and the macrophage–trophoblast interaction in pregnancy and preeclampsia are essential to enhance our knowledge of its modes of action and to evaluate its potential as a therapeutic agent in preeclampsia and other placenta dysfunctional complications of pregnancy.

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Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- Preeclampsia is a pregnancy-related disorder characterized by the maternal syndrome hypertension and proteinuria.
- Inflammatory dysfunction and placental abnormalities lead to the maternal syndrome.
- For a successful pregnancy, a well-balanced macrophage–trophoblast interaction in the placenta is indispensable.

**What New Information Does This Article Contribute?**

- Cluster of differentiation 74 (CD74) is downregulated on placental macrophages.
- CD74 downregulation in macrophages in CD74-knockout mice has a disturbed placental morphology, vascular remodeling, and fetal growth restriction.

Immune cells and their interaction with trophoblasts, including fetal-placental macrophages and villous trophoblasts, facilitate placental growth by a well-balanced regulation. Placental macrophages are of fetal origin and play a direct role in early placental development. CD74 is crucial for biogenesis and triggers inflammatory protein expression and modulation of cell survival. Here, we show a downregulation of the CD74 expression and presentation on placental macrophages in the preeclamptic placenta. Macrophages that lack CD74 show a dysregulated interaction with trophoblasts: adhesion to trophoblasts was diminished and CD74-deficient macrophages exhibited a proinflammatory phenotype when cocultured with trophoblasts. Trophoblasts activated by proinflammatory stimuli expressed more vasoactive and antiangiogenic factors. CD74-/- mice show altered placenta morphology with impaired spiral artery remodeling leading to fetal growth restriction. Our results demonstrate a novel link between preeclampsia and the regulatory interaction of placental macrophages and villous trophoblasts with a specific role for CD74. Understanding the early regulation of placenta development and the interaction of immune cells with trophoblasts as the main cell population is crucial for the understanding of the development of placental anomalies that lead to preeclamptic syndrome and likely also other pregnancy disorders, such as intrauterine growth restriction and preterm delivery.
CD74-Downregulation of Placental Macrophage-Trophoblastic Interactions in Preeclampsia

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SUPPLEMENTAL MATERIAL

Online Methods

Patients

Human placenta, decidua and blood sampling was approved by the Regional Committee of Medical Research Ethics in Eastern Norway and the Medical Faculty of Charité Berlin. Placental biopsies (from a centrally located placental cotyledon, avoiding maternal decidua) were obtained following cesarean sections from 50 preeclamptic women (PE) and 28 women with normotensive and uncomplicated pregnancies. Decidual tissue was collected through vacuum suctioning of the placental bed.\(^1\) The PE group was divided into early onset PE (<34 gestational week, n=26) and late onset PE (≥34 gestational week, n=24). The uncomplicated pregnancy group consisted of healthy, normotensive women undergoing cesarean section due to breech presentation or other reasons. Patient characteristics are shown in Online Table I.

Animals

Local authorities (LaGeSo, Berlin, Germany) approved all experiments. Primary cultures of macrophages were generated from the bone marrow of male 10-12 week old wild-type (C57Bl/6JOlaHsd) mice; Harlan Laboratories, Rossdorf, Germany) or CD74 knockout (B6-(Cd74)tm) mice. Mice were fed normal chow diets (#V1124-300, Ssniff, Soest, Germany) and housed under standardized light-dark cycles and SPF conditions. Mice were observed daily in order to minimize harm. To evaluate the preeclamptic phenotype, wild-type (C57Bl/6JOlaHsd) and CD74 knockout (B6-(Cd74)tm) mice were bred (plug-recognition day was assigned as day 1) and transferred to metabolic cage on day 16 of pregnancy for 24 h. Blood pressure was monitored by telemetric bloodpressure measurement. All experimental animals were sacrificed on day 18. Fetuses and placentas were counted and weighted. Urinary mouse albumin was measured with a commercially available ELISA (CellTrend, Germany).

Doppler ultrasound studies were performed on day 15/17 as described previously.\(^2\) The animals were anesthetized with 1.5% isoflurane via an oxygen mask. Maternal heart rates and rectal temperatures were monitored (Model THM100, Indus Instruments). Rectal temperature was maintained at 36°C to 38°C. The hair was removed from the abdomen, and prewarmed gel was used as an ultrasound-coupling medium. The pregnant mice were imaged with an ultrasound biomicroscope and a 30-MHz or 40-MHz transducer at 30 frames per second (Model Vevo 660, VisualSonics Inc). The Doppler waveforms were obtained in the proximal uterine artery, the distal uterine artery (distal of the main branch of the uterine artery between 2 embryonic implantation sites) and the embryonic umbilical artery. Peak systolic velocity (PSV) and end-diastolic velocity (EDV) were measured from 3 consecutive cardiac cycles that were not affected by motion caused by maternal breathing, and the results were averaged. The resistance index (RI=(PSV−EDV)/PSV) was calculated. For biometric measurements, abdominal and head transversal and longitudinal diameters were measured, and the circumference was calculated.

For Two-cell stage embryo transfer, 9-12 weeks old female CD74 knockout (B6-(Cd74)tm) mice or wild-type C57Bl/6JOlaHsd mice were treated (i.p.) by 5 IU Pregnant-Mare-Serum-Gonadotropin (Intergonan 240 I.E./ml, Intervet). After 48 hours, mice were injected (i.p.) with 5 IU human chorionic gonadotropin (Ovogest
and mated by male CD74 knockout (B6-(Cd74)tm) mice or wild-type C57Bl/6JOlaHsd. Two-cell stage embryos were flushed with EmbryoMax M2 Medium (MR-015-D, Merck Milipore) from the oviduct after cervical dislocation of the female donor mice 1.5 days after mating. 8-12 two-cell stage embryos were transferred by embryo transfer Pipette under stereomicroscope into the oviduct of an anesthetized pseudopregnant wild-type recipient (day 0.5 of pseudopregnancy). Mice were sacrificed on day 18 of pregnancy. Fetuses and placentas were counted and weighted.

**Isolation of primary cells from human placenta**

All placentas were processed within 2 h after delivery. Whole placentas were placed on ice in 0.9% (w/v) NaCl buffer, dissected/removed from fetal membranes, basal plate, umbilical cord and fibrotic tissue, and villous tissue was minced and washed several times, to minimize blood contamination. Then, various trypsin (Sigma) and DNase I (Roche) digestion steps were used and supernatants were filtered and collected in New Born Calf Serum (Biochrome). For Hofbauer cell isolation supernatant was discarded and undigested tissue was digested further with collagenase A (Roche) and DNase I. Cell suspensions were loaded on Percoll (GE Healthcare) gradual gradients. Cells were collected (Hofbauer cells: 35-45% gradient; trophoblasts: 40-50% gradient; general cells: 70% gradient) and washed. For the general cell population protocol, cells were directly processed for flow cytometry. For primary trophoblasts, cells were negatively immunopurified using HLA-ABC (DakoCytomation) and magnetic dynabeads (Dynal Biotech), for Hofbauer cell isolation, cells were purified by an adhesion step (culturing on a plastic dish for 30min).

**Macrophage preparation and cell culture**

Macrophages were generated from two different origins: Human peripheral blood mononuclear cells (PBMCs) and mouse bone marrow-derived (BMD). Nomenclature and experimental design of *in vitro* differentiated and activated macrophages follows the guidelines described by Murray et al.3

PBMCs derived macrophages: 80 ml of blood were drawn from human healthy donors. The study was approved by the Regional Committee of Medical Research Ethics (Charité). PBMCs were purified by Ficoll gradient centrifugation. To enrich monocyte yield, an adhesion step was performed by seeding the cells (3.5*10^5 cells/cm²) in serum-free RPMI1640 medium (Sigma) containing 10mM HEPES (Gibco), 50 μM β-mercaptoethanol (β-ME) (Sigma) and 1% (v/v) penicillin/streptomycin (P/S) (Gibco) for 2 h. Cells were washed and cultivated in basal RPMI containing 20% fetal calf serum (FCS) (v/v) (Biochrom), 10 mM HEPES, 50 μM β-ME, 1% (v/v) P/S media with M- colony stimulating factor (CSF) (100ng/ml) for 6 days with media change every 3 days to reach the M(-)status (also known as M0). For further activation of M(-) into M(IL-4) (also known as M2) cells were cultured in basal medium with IL-4 (20 ng/ml) for 18 h. All cytokines were bought from Active Bioscience (Germany).

BMD macrophages: Cells were isolated from the femur and tibia of freshly euthanized mice, by flushing with ~10 ml of activation media: RPMI1640 containing L-glutamine (Gibco), 10% (v/v) FCS, 10 mM HEPES, 50 μM β-ME, 1% (v/v) P/S, without CSF-1. Cells then were pelleted, and resuspended into monocyte differentiation media: DMEM (Gibco), 10 % (v/v) FCS, 5% (v/v) adult horse serum (Cell Concepts), 1:100 non-essential amino acids (Sigma), 50 μM β-ME (Sigma), with 20% (v/v) L929 conditioned media containing CSF-1 /RPMI media. Conditioned
media containing CSF-1 was generated by collecting the media from L929 cells (ATCC) cultured for 14 d in DMEM containing 10% (v/v) FCS, 1:100 non-essential amino acids, 10 mM HEPES and 1% (v/v) P/S. For macrophage differentiation, 10 x 10^6 bone-marrow derived cells were cultivated in 50 ml of differentiation media for 7 d in sealed, hydrophobic Teflon® bags (FT FEP 100 C (DuPont), American Durafilm) at 37°C and 10% CO₂. The yield of BMD M(-) macrophages (also known as M0) from one bag was consistently ~70-100 x 10^6 cells with a purity of >95% (determined as F4/80+ CD11b+ cells by flow cytometry, data not shown). For activation of M(-) into M(IL-4+IL-13) (also known as M2) or M(LPS) (also known as M1), BMD M(-) cells were harvested from Teflon bags, pelleted and resuspended into activation media containing recombinant mouse IL-4 (10 ng/mL) and IL-13 (10 ng/ml) or LPS (100ng/ml). For analysis by real-time RT-PCR and Western blotting, 2 x 10^5 BMD M(-) and BMD M(IL-4+IL-13) cells were plated per well in 6-well plates. In all cases, the macrophages were first allowed to rest and adhere for 2 h. Unless otherwise noted, cells were activated for 24 h at 37°C and 5% CO₂.

SGHPL-4 cells derived from primary human first trimester extravillous trophoblasts (EVT) transfected with the early region of SV40, known previously as MC418, were a kind gift from Judith E. Cartwright (St. George’s University of London, London, United Kingdom). These cells show similar invasive capabilities to primary EVTs and retain features of normal EVTs. SGHPL-4 cells were cultivated in HAM’s F10 (Biochrom) media containing 10% (v/v) FCS and 1% (v/v) P/S.

1 x 10^6 BMD M(IL-4+IL-13) and 2 x 10^5 SGHPL-4 cells were cultivated separately or together in HAM’s F10 media containing 1% (v/v) FCS and 1% (v/v) P/S for 48 h. Media was collected and stored.

Endotoxin-free recombinant human MIF was purified as previously described. Prior to stimulation protocol SGHPL-4, Hofbauer cells and Blood-derived macrophages were cultivated in starvation media (basal media supplemented with 0.1% FCS) for 18 hrs. Cells were treated with varying concentrations (10, 50, 200, 200 ng/ml) of MIF. After indicated time (5, 10, 15, 30 min) cells were washed with cold PBS and proteins were isolated on ice using RIPA buffer supplemented with Complete protease inhibitor cocktail (Roche Diagnostics) and phosphatase inhibitory cocktail (Sigma). 100 μg of protein lysates were loaded on SDS-PAGE. Cells treated with EGF in concentration of 10 ng/ml for 10 min (Hofbauer cells) and 15 min (SGHPL-4) were used as a positive control. For expression analysis, starved SGHPL-4 cells were stimulated with 50 ng/ml MCP-1, 200 ng/ml CCL5 (Miltenyi Biotec) and 10ng/ml TNFα (Sigma-Aldrich) or in combination of all three cytokines for 6h or 24h as indicated.

**mRNA isolation, real-time RT-PCR and microarray analysis**

Total mRNA was isolated from tissues (homogenized by ceramic balls) and cells using QIAzol lysis reagent and Qiagen RNeasy mini kit (Qiagen) with on-column deoxyribonuclease I step (Qiagen) according to manufacturer’s protocol. mRNA quality and concentration was measured by NanoDrop-1000 spectrophotometer (Peqlab). 2 μg of mRNA was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time polymerase chain reaction (PCR) was detected on ABI 7500 Fast Sequence Detection System (Applied Biosystems) and analyzed by 7500 Fast System Software (Applied Biosystems). Primers and probes (Online Table II) were designed with PrimerExpress 3.0 (Applied Biosystems) and synthesized by Biotez (Germany). Expression of target genes was normalized to 18s expression.
Microarray analysis of human placenta and decidua from the Oslo Pregnancy Biobank was described earlier. Microarray analysis of M(-) and M(IL-4) was done with the Illumina HumanHT-12_V3_0_R2 according the Minimum Information About a Microarray Experiment (MIAME) criteria. Data is provided at ArrayExpress under accession number E-MTAB-3309. Differentially expressed genes (65% FDR score) were investigated through the use of QIAGEN’s Ingenuity® Pathway Analysis (IPA®, QIAGEN).

Western blot
Placental tissue or cells were lysed with RIPA buffer. Protein concentration was measured using Bradford reagent (ROTH) and certain amounts of denaturized protein samples were separated on SDS-PAGE. Further, proteins were transferred to nitrocellulose membrane using semi-dry blotting. Unspecific binding was reduced by 3% w/v BSA, while hCD74- (abcam), β-actin- (CellSignaling), eIF4 - (CellSignaling) and mCD74- (R&D) specific antibody incubations were carried out overnight at 4°C. Secondary antibodies were conjugated with fluorochrome (IRDye800CW), excited and documented on ODYSSEY infrared scanner (LI-COR Biosciences). For loading control, membranes were stripped and re-incubated.

Flow cytometry and high-throughput sampler (HTS)
Placental cells were kept on ice in washing-buffer (PBS containing 0.1% FCS). 1 x 10⁶ cells were stained for extracellular markers (CD14-PE-Cy7, CD74-PE, BD Bioscience), fixed and cell membrane was permeabilized using Fix/Perm buffer (BD Biosciences). Then, Fc receptor blocking was performed (eBioscience) and cells were incubated with antibodies against intracellularly expressed proteins (cytokeratin 7, Abcam). Incubation with secondary antibody was performed (anti-rabbit-APC, ImmunoResearch). 1 x 10⁵ events were recorded on BD FACSCanto II system using BD FACSDiva software (BD Biosciences). Data was analyzed using FlowJo software (TreeStar Inc.). Positive gating coordinates were set according to isotype controls.

Trypsinized cells after co-culture experiments were loaded onto 96-well plates and analyzed on high throughput sampler. 100 µl of cell suspension was sampled and the amount of cells was recorded and counted on BD FACSCanto II system using BD FACSDiva software. Data was analyzed using FlowJo software and is presented as absolute number of cells in SGHPL-4 cell gate (coordinates set according to forward scatter (FSC) and side scatter (SSC) of SGHPL-4 single culture).

Immunostaining
Human control placental sections were deparaffinized in xylene followed by rehydration through graded ethanol. Antigen was retrieved by heat-induced epitope retrieval (HIER) method in 0.01 M citrate buffer pH 7. Non-specific antibody binding was blocked with Ultra V Block (Lab Vision). Double immunostaining of mouse anti-CD74 (abcam) and rabbit anti-cytokeratin 7 (abcam) or rabbit anti-CD163 (DB Biotech) was performed and secondary antibody goat anti-mouse Alexa555 and goat anti-rabbit Alexa488 was applied. After extensive washes with PBS/T cell nuclei were stained with DAPI.

Paraffin embedded mouse placental tissue was cut into 4 µm histological sections at the mid sagittal plane using a microtome (Leica). Tissue sections were deparaffinized, rinsed in distilled water, and dehydrated twice in ethanol 70 %. Masson-Goldner trichrome staining kit (VWR international) was used to visualize the morphologically different areas of placental tissues. Briefly, tissue sections were
stepwise stained with Weigert’s iron hematoxylin, Azophloxine staining solution and phosphotungstic acid Orange G, and Light green SF solution following manufacturer’s instructions. Finally, the tissue was dehydrated and mounted. Image acquisition was performed using a slide scanner (Mirax Midi, Zeiss). The size of placental labyrinth and junctional zone was assessed based on morphological criteria and quantified using the program MiraxViewer. The ratio between the labyrinth and junctional zone (L/Jz ratio), which is considered as a marker for placental function, was calculated by dividing the size of the labyrinth by the size of the junctional zone. Additionally, parallel sections were stained with Periodic Acid Schiff (PAS; for general morphological evaluation and detection of fibrinoid) and α-actin (marker for vascular smooth muscle cells, VSMC) (DAKO M851) with MOM-Kit (Vector Laboratories) for investigating spiral artery remodeling. The Protocol was described before. Briefly, we used Depex mounting medium (VWR international GmbH), Aqua-Poly/Mount (Polysciences, Inc.), and non-conjugated goat anti-mouse immunoglobulins (Acris Antibodies) at a final dilution 1:50. Image acquisition was performed using Zeiss microscope Axio Imager M2 with AxioVision 4.1.

Spiral artery cross sections within the mesometrium were investigated for remodeling process. The loss of smooth muscle cells (α-actin) was detected and evaluated by % of circumference. We interpreted 100% (representing total loss of smooth muscle cells) as vascular remodeling of the spiral artery. Area and perimeter of spiral arteries were also measured using AxioVision 4.1.

Soluble CD74-, MIF-ELISA, sFlt1-, PLGF- and cytokine/chemokine analysis

Determination of MIF levels were performed with an ELISA technique as previously described, using capture antibody MAB289 and detection antibody BAF289 (R&D, Germany). The measurement of circulating CD74 was performed using a competitive sandwich ELISA as previously described. As capture antibody anti-CD74 (clone C-16, Santa Cruz, SC-5438, 250ng/ml) was used and for detection clone LN-2 (Santa Cruz, sc-6262, 400ng/ml). The rhCD74 protein standard used for the ELISA was purchased from R&D systems (3590-CD). The inter-assay variability was 25% and the assay sensitivity was 1.56 ng/ml.

For cytokine/chemokine analysis, supernatants were loaded on 96-well plates supplied with Mouse Luminex Screening Assay and procedure was carried out according to manufacturer’s protocol (R&D). Cytokines included in assay were: CCL2, CCL4, CCL5, CXCL1, GDF-15, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-10, IL-17A, II-2, IL-4, IL-6, TNF-α and VEGF. Data was recorded and analyzed on Luminex 200 System using Bio-Plex Manager software 6.0 (Bio-Rad).

Serum PLGF and sFlt1 were analyzed on Elecsys (Roche Diagnostics). sFlt1/PLGF ratios were calculated.

Silencing RNA (siRNA)

For silencing procedure, human M(-) and M(IL-4) were cultivated in RPMI1640 media w/o P/S and w/o M-CSF. Transfection was carried out according to manufacturer’s protocol using DharmaFECT 1 transfection reagent and ON-TARGETplus Non-targeting Pool siRNA (NT) as control and SMARTpool: ON-TARGETplus CD74 siRNA (siCD74) (Thermo Scientific) all in a final concentration of 50 nM. After 24 h, media was changed to RPMI1640 containing 20% (v/v) FCS, 10mM HEPES, 50 μM β-ME, 1% (v/v) P/S to reduce cytotoxicity. Experiments were terminated after 48h with mRNA or protein extraction.
Adhesion assay

Human M(IL-4) were stained with CellTracker Green CMFDA Dye (Life technologies) for 45min in 37°C in RPMI1640 containing 10mM HEPES, 50 μM β-ME, 1% (v/v) P/S. After additional 30min in the same media without CellTracker, cells were detached from culture dish using Trypsin-EDTA (Sigma). Cells were suspended to cell concentration of 5 x 10⁵ / 200 µl in serum-free media. Cells were then added onto SGHPL-4 cell layer formed by overnight culture of 5 x 10⁵ cells in 96-well plate format. After 8 h non-adherent macrophages were washed out and adherent cells were documented with fluorescence microscopy and AxioVision software (Carl Zeiss MicroImaging). Afterwards, cells were lysed using 0.1M NaOH for 30 min in 37°C and fluorescence was measured on microplate reader (TECAN).

Statistics

Data are presented as mean ± SEM when normally distributed or as median with interquartile range when non-normally distributed (or as indicated). Normal distribution was assessed by Kolmogorov-Smirnov test. Groups were compared using the unpaired t test, Mann–Whitney U test, 1-way ANOVA, or Kruskal-Wallis test as appropriate and indicated in the figure legends. Multigroup comparisons were followed by post hoc testing, including the Scheffe test, Dunnett T3, and Mann–Whitney U tests with a Bonferroni correction. p<0.05 was considered as statistically significant.
**Online Tables**

**Online Table I. Clinical characteristics of expression studies subjects.**

<table>
<thead>
<tr>
<th>Characteristics at delivery</th>
<th>Normotensive controls (n=28)</th>
<th>Early onset Preeclampsia: Delivery &lt;34 GW</th>
<th>Late onset Preeclampsia: Delivery ≥ 34 GW</th>
<th>P value (Control vs early onset PE and control vs late onset PE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>31.2 ± 4.2</td>
<td>30.7 ± 5.2</td>
<td>32.5 ± 6.0</td>
<td>0.7 and 0.4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.6 ± 3.4</td>
<td>30.5 ± 4.0</td>
<td>32.6 ± 6.2</td>
<td>0.06 and 0.007</td>
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<tr>
<td>Gestational weeks</td>
<td>39.0 ± 0.9</td>
<td>30.7 ± 2.3</td>
<td>36.9 ± 1.6</td>
<td>&lt;0.001 and &lt;0.001</td>
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<tr>
<td>Blood pressure Systolic (mm Hg)</td>
<td>119 ± 11.6</td>
<td>168 ± 17.1</td>
<td>160 ± 14.5</td>
<td>&lt;0.001 and &lt;0.001</td>
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<tr>
<td>Blood pressure Diastolic (mm Hg)</td>
<td>72.1 ± 10.9</td>
<td>102.3 ± 7.4</td>
<td>99.9 ± 5.8</td>
<td>&lt;0.001 and &lt;0.001</td>
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<tr>
<td>Baby weight (g)</td>
<td>3492 ± 402</td>
<td>1372 ± 415</td>
<td>3035 ± 681</td>
<td>&lt;0.001 and 0.007</td>
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</table>

Clinical characteristics of the preeclamptic cases and controls from the Oslo study population. Data are presented as mean ± standard deviation, BMI: body mass index.
<table>
<thead>
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<th>Sequence</th>
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<td>18S</td>
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<td>5'-GAGCCAGTTTGCAGAAGGTAG-3'</td>
<td>5'- ATCCGACTCAGGGGTCAATG -3'</td>
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</table>
Online Figure I. CD74 in decidua, soluble CD74 (sCD74), macrophage migration inhibitory factor (MIF) in circulation of preeclamptic women (PE) and correlation of CD74 expression and (anti)-angiogenic factors. A) CD74 expression was not altered in deciduas of PE compared to healthy women (control; n=28). PE is subdivided in early onset PE (delivery <34 week of gestation; grey bar; n=26) and late onset PE (delivery ≥34 week of gestation; black bar; n=24). B) sCD74 was lowered in early PE and late PE compared to control, although not statistically significant. C) Accordingly, MIF showed a trend to be enhanced both in early PE and late PE, although not statistically significant. D) Placental CD74 expression significantly correlated to the serum sFlt1/PLGF ratio in the PE group but not in controls. Correlation of placental CD74 expression to serum sFlt1 and PLGF is also shown.
Online Figure II. CD74 expression in human placenta and different cell types. CD74 expression normalized to 18s was high in primary Hofbauer cells isolated from placenta (n=5), and in in vitro differentiated and activated human PBMC-derived macrophages (M(-) and M(IL-4)), intermediate in placenta (n=4) and low in primary trophoblasts isolated from placenta (n=5) and in a first trimester derived trophoblast cell line (SGHPL-4) (n=3).
Online Figure III. Phospho-ERK1/2 (pERK) signaling in SGHPL-4, Hofbauer cells and blood derived macrophages. Stimulation by macrophage migration inhibitor factor (MIF) of Hofbauer cells and blood-derived macrophages led to an activation of pERK (phosphorylation of p44 and p42). SGHPL-4 cells did not show an activation by MIF stimulation. Epidermal growth factor (EGF) served as positive control.
Online Figure IV. Characterization of CD74 in CD74 silenced macrophages. A) CD74 expression normalized to 18s was lowered by CD74 silencing (siCD74) in \textit{in vitro} differentiated and activated human PBMC derived macrophages (M(IL-4)) compared to control (NT) (n=27 each; ***p<0.001; Mann Whitney test). B) CD74 protein was lowered after 48h and 72h in M(IL-4)siCD74 vs. M(IL-4)NT. eIF4 was used as a loading control.
Online Figure V. Ingenuity Pathways Analysis (IPA) summary. A) Top ten canonical pathways and (B) top ten diseases and biological functions influenced by CD74 down-regulation in M(IL-4) macrophages are shown. The ratio is calculated as a number of genes from the dataset that map to the pathway divided by the number of total genes included into the pathway. The yellow line represents the threshold of p<0.05 as calculated by Fischer's test.
Online Figure VI. Adhesion molecule expression in CD74 silenced macrophages. In *in vitro* differentiated and activated human PBMC derived macrophages (M(IL-4)) CD74 silencing (siCD74) led to a downregulation of ALCAM, ICAM and SDC2 expression (normalized to 18s) when compared to control (NT) (n=27 each; **p<0.01, ***p<0.001; Mann Whitney test).
Online Figure VII. Characterization of CD74 in bone-marrow derived (BMD) macrophages of CD74-knockout (CD74-KO) mouse. *In vitro* differentiated and activated BMD macrophages (M(IL-4+IL-13)) showed high CD74 expression (normalized to 18s) when derived from WT but no expression when derived from CD74-KO mice (n=6 each; ****p<0.0001; Unpaired t test).
Online Figure VIII. Characterization of CD74 in placenta of CD74-knockout (CD74-KO) mouse. CD74 was absent in placentas of CD74-KO mice. Wild-type (WT) mice showed robust CD74 protein level.
Online Figure IX. Labyrinth zone CD74<sup>−/−</sup> mouse. Size of labyrinth zone was not changed in CD74<sup>−/−</sup> (n=13) in comparison to WT (n=17).
Online Figure X. Spiral arteries in CD74⁻/⁻ mouse. A) Representative picture of a placenta stained with α-actin. M=mesometrial tissue; D=decidua basalis; T=trophospongium; L=labyrinth. B) Area and perimeter of spiral arteries localized in mesometrial tissue and decidua basalis were lowered in CD74⁻/⁻ (n=24) compared to WT (n=37). M: CD74⁻/⁻ (n=86); WT (n=95). D: CD74⁻/⁻ (n=24); WT (n=37). *p<0.05, **p<0.01, ***p<0.001; T test).
Online Figure XI. Resistance Index (RI) of Uterine arteries and umbilical artery in CD74-/- mice. A) Representative pictures of the flow in the distal uterine artery in WT and CD74-/- mice measured by Doppler ultrasound. B) Peak systolic velocity (PSV) and end-diastolic velocity (EDV) were measured in proximal uterine artery, distal uterine artery and embryonic umbilical artery and the resistance index (RI = (PSV–EDV)/PSV) was calculated. RI was increased in uterine distalis of CD74-/- mice compared to wild type (WT) on day 15/17 of pregnancy. RI of the proximal uterine artery and umbilical artery was unchanged (n=10 each; *p<0.05; Mann Whitney test).
Online Figure XII. Characterization of maternal syndrome in CD74^{−/−} mice. Mean arterial pressure (MAP) (n=6 each) and albuminuria (n=4 each) did not change in CD74^{−/−} vs. wild type (WT).
Online Figure XIII. Fetal growth restriction and lowered placenta weights of CD74<sup>−/−</sup> fetuses developed in WT foster mothers. Pup and placental weights resulting of CD74<sup>−/−</sup> derived two-cell stage embryos transferred into WT mice (n=14) were lower on day 18 of pregnancy when compared to WT derived embryos (n=15). *p<0.05; unpaired t-test.
Online References


