Chapter 4

Syncytiotrophoblast extracellular vesicles from healthy and preeclamptic placentae induce monocyte and granulocyte activation

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Abstract

Pregnancy is characterized by a systemic inflammatory state of the maternal body. Amongst others, this state features an increase of numbers and activation of monocytes and granulocytes as well as a shift of CD16- classical monocytes towards CD16+ intermediate monocytes. During the pregnancy-complication preeclampsia, this inflammatory state is exaggerated, monocytes and granulocytes are even more activated, and the shift of CD16- classical monocytes towards CD16+ intermediate monocytes partially goes further to CD16++ non-classical monocytes. Syncytiotrophoblast extracellular vesicles (STB EV) are secreted from the placenta during the whole pregnancy and their plasma concentration is even elevated during preeclampsia. It is believed, that syncytiotrophoblast exosomes are rather tolerance-inducing while syncytiotrophoblast microvesicles (STB MV) are rather activating and therefore are triggering preeclampsia. However, their functional involvement in the inflammatory state of pregnancy and preeclampsia remains partially elusive. Therefore, in this study, we aimed to study the effects of STB EV from normal and preeclamptic placentae on monocytes and granulocytes and compared the function of STB MV and exosomes to each other. STB EV from normal placentae induced activation of monocytes and granulocytes and monocyte maturation into CD16+ intermediate monocytes. Also, STB EV from preeclamptic placentae induced activation of monocytes and granulocytes and monocyte maturation, but only at a similar level than STB EV from normal placentae did. STB MV and exosomes had similar effects, but the effect of exosomes was stronger. Therefore, we concluded that STB EV may be involved in mediating the activation of monocytes and granulocytes, and therefore the systemic inflammatory state during normal pregnancy. Other factors may be responsible for the exaggerated inflammatory state during preeclampsia. Furthermore, the stronger effect of exosomes compared to STB MV may be based on a differential molecular load of both STB EV fractions.
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Introduction

The placenta has an important function in pregnancy, not only in the exchange of nutrition, gasses and waste products between mother and fetus, but also in adapting the maternal physiology to pregnancy.[1,2] To do so, the placenta produces various factors, such as hormones, cytokines and syncytiotrophoblast extracellular vesicles (STB EV). STB EV are membrane-coated vesicles released from the syncytiotrophoblast into the maternal blood, where they can reach their target maternal cells.[1,3] In general, extracellular vesicles can be subdivided into several classes [4,5], but placental STB EV are mainly subdivided into syncytiotrophoblast microvesicles (STB MV), and exosomes.

STB MV are 200 nm to 1000 nm in size and are shed from the apical plasma membrane of the syncytiotrophoblast. Exosomes are nanovesicular structures of 30 nm to 100 nm in size and are formed in intracellular multivesicular bodies and released by fusion with the syncytiotrophoblast plasma membrane.[3,6] Both STB MV and exosomes carry a molecular load representative for their placental origin, with which they can affect the function of maternal cells. They share a large portion of this molecular load, however, both vesicle fractions also show distinct markers which suggests that a part of their proteome is unique to either STB MV or exosomes.[3,6,7] Therefore, STB MV and exosomes are thought to have different functions.

It has been shown, that the release of STB EV is increased during pregnancy-complications such as preeclampsia (PE).[8] PE is a symptom complex affecting 2 – 5 % of pregnancies and causing the death of approximately 76,000 women and 500,000 infants per year worldwide.[9] PE is characterized by new-onset hypertension and proteinuria in the second half of pregnancy.[10] A shallow placentation and insufficient remodeling of spiral arteries may cause hypoxia and reperfusion injury in the placenta which is thought to increase the secretion of diverse placental factors, such as STB EV, that in turn lead to an activation and dysfunction of the endothelium and systemic inflammation in the maternal body.[10] Not only the number of STB EV is increased in preeclampsia, it has also been suggested that the molecular load of STB EV from preeclamptic placentae differs from that of STB EV from normal placentae,[11] which may indicate an altered function of STB EV during PE.

The immune response is one of the physiological functions that is largely altered in pregnancy and even more in PE. Changes have been shown in both the adaptive and the innate immune response.[12–15] Monocytes and granulocytes are a part of the innate immune response and have been shown to be both increased in number and phenotypically activated during pregnancy, characterized by upregulation of activation markers such as CD11b.[13,16] According to their expression of the lipopolysaccharide receptor (CD14) and of the low affinity Fc receptor (CD16), monocytes can be subdivided into classical (CD14++ CD16-), intermediate (CD14++CD16+) and non-classical (CD14+CD16+).[17] During pregnancy,
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a shift from the classical towards the intermediate subsets has been observed. In PE, these effects have been described to be even more pronounced than in normal pregnancy. Here, intermediate monocytes are even more increased compared to non-pregnant status and normal pregnancy and monocytes and granulocytes are activated stronger than in normal pregnancy. It is believed, that placental factors or direct contact of monocytes with the placenta are responsible for the activation during pregnancy and PE.

In the present study, we questioned whether STB EV from healthy pregnant women are able to activate monocytes and granulocytes in vitro and to induce a shift from classical monocytes towards intermediate monocytes. Since STB MV and exosomes may have different functions, we studied the effect of STB MV and exosomes separately. Secondly, we tested the hypothesis that STB MV and exosomes from preeclamptic placentae have a different function from normal pregnant STB MV and exosomes.
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Materials Methods

**Ex vivo placenta perfusion**

Experiments with placental tissue were approved by the ethics committee of the University Hospital Jena, Germany. Four placentae from healthy pregnancies and two placentae from preeclamptic women were collected after informed consent immediately after vaginal delivery or elective caesarian section. A proper cotyledon was chosen macroscopically and *ex vivo* double-sided placenta perfusion commenced within 20 min after receipt of the placenta. Perfusion suspension was composed of 1 l NCTC-135 (with L-glutamine, without phenol red, without vitamin B12, without sodium hydrogen carbonate, AppliChem GmbH) with 0.5 l Earl’s buffer, 60 g bovine serum albumin (MP Biomedicals LLC), 15 g Dextran (FP 40, SERVA electrophoresis GmbH), 2 g D-Glucose (water-free, Merck KGaA), 0.38 g Amoxicillin (Sigma-Aldrich Chemie GmbH) and 0.75 ml Heparin (Heparin-Natrium-25.000, 25,000 i.E./5 ml, Rathiopharm Gmbh). Earl’s buffer contained 111.225 mM NaCl (Carl Roth GmbH + Co.KG), 5.365 mM KCl (Merck KGaA), 1.015 mM NaH2PO4 (Merck KGaA), 26.187 mM NaHCO3 (Merck KGaA), 811.425 µM MgSO4*7 H2O (Merck KGaA), 1.36 mM CaCl2*2 H2O (Merck KGaA). The perfusion suspension was filtered using a 0.8/0.2 µm filter (AcroPak™ 200 Capsules with Supor® Membrane, Pall Corporation), adjusted to pH = 7.4 with NaOH (Carl Roth GmbH + Co.KG) and frozen to -20 °C until *ex vivo* perfusion of an isolated placental cotyledon.

To perfuse the fetal circulation, cannulas were introduced into the fetal main vein and artery of the cotyledon and perfusion started with 0.3 mL/min perfusion speed, using perfusion suspension, which was purged with 95 % nitrogen / 5 % carbon dioxide. After connecting the maternal circulation, the flow was slowly increased to its final speed at 3 mL/min to avoid pressure peaks. To perfuse the maternal circulation, the cotyledon was put into a perfusion chamber and cannulas were introduced into the intervillous space by cautious penetration of the decidua. The maternal circuit was perfused at 12 mL/min and perfusion suspension was purged with technical air. After a 30 min rinsing period, perfusion was performed for 120 min, the perfusion suspension was collected and centrifuged for 10 min at 380 g at room temperature (RT) to pellet cells. The supernatant was frozen at -80 °C until separation of STB MV and exosomes.

**Quantification of STB EV**

We aimed to stimulate blood samples with STB MV and exosomes with a concentration present in plasma from late pregnant women (36 +/-1 weeks of gestation). Recently, we quantified the peripheral STB EV concentration (consisting of STB MV and exosomes) in plasma from women at 36 (+/-1) weeks of gestation (manuscripts in preparation, see chapter 3 of this thesis) using the enzyme-linked
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Sorbent assay as previously described. We assume that the ratio of STB MV to exosomes is similar in plasma from late pregnant women and the perfusion suspension. STB EV were quantified in the supernatant of the perfusion suspension using the enzyme-linked sorbent assay as previously described. The concentration of STB EV was adjusted to the concentration in late pregnant plasma for the stimulation of whole blood with perfusion-derived STB MV or exosomes.

Separation of syncytiotrophoblast microvesicles and exosomes from perfused perfusion suspension

To pellet cell debris, 38 ml of the perfusion suspension was centrifuged for 10 min at 10.000 g at 4 °C (Figure 1). All following centrifugations were performed in ultra-clear centrifuge tubes (Beckman Coulter, Krefeld, Germany). STB MV were pelleted for 30 min at 18.900 g at 4 °C and the supernatant was filtered with a 0.8/0.2 µm double filter (Acrodisc PF 32 mm Syringe Filter with 0.8/0.2 µm Supor Membrane, Pall Corporation, Port Washington, NY, USA). Exosomes were pelleted from the filtrate by ultra-centrifugation for 70 min at 100.000 g at 4 °C to pellet exosomes. The STB MV pellet and exosome pellet were each washed in 1 % BSA (Fraction V, MP Biomedicals LLC, Solon, OH, USA) in 0,05 % Tween20-PBS (Polyoxyethylene(20)-sorbitan-monolaurate, Dulbecco’s phosphate buffered saline; both Sigma-Aldrich Chemie GmbH, Hamburg, Germany) and pelleted again for 30 min at 18.900 g at 4 °C or for 70 min at 100.000 g at 4 °C, respectively. Pellets were re-suspended in 200 µl of 1 % BSA in 0,05 % Tween20-PBS (pH = 7,4 Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA), aliquoted and stored at -80 °C until usage.

Figure 1: Centrifugation protocol for the isolation of STB MV and exosomes from suspension after ex vivo placenta perfusion
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Blood stimulation and flow cytometric analysis

This study was approved by the medical ethical committee of the University Medical Centre Groningen. Blood samples for stimulation were collected after informed consent at day 5, 6 or 7 of the current cycle from healthy nulligravida between 18 and 40 years of age, with a body-mass-index between 18 and 30. Exclusion criteria were smoking, usage of an intra-uterine device, infertility, immune-related disorders, flu-like symptoms within four weeks before blood donation, and use of medication other than folic acid or oral contraceptives. The blood was collected in lithium-heparin tubes (Lithium-Heparin, BD Vacutainer, LH 170 I.U., BD, Plymouth, United Kingdom).

STB MV and exosomes from each placenta were used to stimulate blood from 3 different donors for 24 h at 37 °C, 5 % CO₂. Blood was aliquoted into 12 well plates at 3 ml per well and stimulated with STB MV or exosomes adjusted to the peripheral plasma concentration in late pregnancy (see Quantification of STB EV). Since STB MV and exosomes were re-suspended in 1 % BSA (Sigma-Aldrich Chemie GmbH) in 0,05 % Tween20-PBS (Phosphate-buffered saline, pH = 7,4 Gibco, Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States of America) equivalent volumes of 1 % BSA in 0,05 % Tween20-PBS were applied as control.

After the stimulation, whole blood was diluted in an equivalent volume of RPMI 1640 medium (Gibco, Thermo Fisher Scientific Inc., Waltham, Massachusetts, United States of America) and red blood cells were lysed by two times incubation in ammonium chloride buffer (155.17 mM NH₄Cl, 9.99 mM KHCO₃, 0.56 mM C10H18N2Na2O10, Clinical Pharmacy, University Medical Center Groningen, Groningen, The Netherlands) at 10 mL per 1 mL whole blood for 10 min at 4 °C. All centrifugation steps were performed for 4 min at 4 °C at 560 g. Cells were re-suspended in wash buffer (PBS with 2 % fetal calf serum, Greiner Bio One International GmbH, Alphen a/d Rijn, The Netherlands), filtered (Falcon® 5mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap, 35 µm nylon mesh, Corning, Corning, NY, USA) and counted with a TC20™ Automated Cell Counter (BioRad, Hercules, California, United States of America). Per staining, 1,000,000 cells were blocked in wash buffer with 20 % rat serum (Normal Rat Serum, Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, United States of America) for 20 min at 4 °C. Next, cells were stained in the dark for 30 min at 4 °C in 50 µl of the antibody mix or an isotype mix at the same antibody concentration: 2.5 µl anti-human-CD14 eFlour450 (clone 61D3, isotype mouse IgG1k), 5 µl anti-human-CD16 PE (clone eBioCB16, isotype mouse IgG1k), 3 µl anti-human-CD115 PE-Cy7 (clone 12-3A3-1B10, isotype rat IgG2αk), 1 µl anti-human-CD66b APC (clone G10F5, isotype mouse IgM), 1 µl anti-human-CD11b FITC (clone ICR44, isotype mouse IgG1k) (all eBioscience, Affymetrix, San Diego, California, United States of America) in wash buffer with 5 % rat serum. Unstained control was incubated in wash buffer with 5 % rat serum only. Flow cytometric analysis was performed on a BD FACSVERSE flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, United States of America).
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America). The gating strategy for flow cytometric analysis was as shown in Figure 2.

**Statistics**

STB EV from each placenta were used to stimulate blood from 3 different donors. Donors differed within the group of healthy placentae \( (n_{\text{placenta}} = 4, n_{\text{donor}} = 12) \) and within the group of PE placentae \( (n_{\text{placenta}} = 2, n_{\text{donor}} = 6) \). To compare the effects of STB MV and exosomes to control and to each other, the non-parametric Friedman test for related samples was run first. Groups (% of gated cells or expression intensity), which reached \( p<0.05 \) in the Friedman test were reanalyzed with the non-parametric Wilcoxon signed rank test for two related samples to compare the treatments: control versus STB MV, or control versus exosomes, or STB MV versus exosomes. P-values below 0.05 were considered significant. Outliers were identified based on their standard score following the rules for critical values of the Grubbs test.

**Figure 2:** Gating strategy for the flow cytometric analysis of monocytes and granulocytes after stimulation with normal syncytiotrophoblast extracellular vesicles

Based on the size (FSC) and granularity (SSC), lymphocytes, monocytes and granulocytes have been identified. Monocytes and granulocytes have been gated together (Monocytes&Granulocytes) and further subdivided according to the expression of the granulocyte marker CD66b and monocyte/macrophage marker CD115. Monocytes have been further subgrouped into classical, intermediate and non-classical monocytes according to their expression of CD14 and CD16. Granulocytes, classical monocytes, intermediate monocytes and non-classical monocytes have been further gated according to their expression of the activation marker CD11b.
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Results

**STB MV and exosomes from normal placentae affect numbers of monocyte subpopulations.**

In control stimulation of whole blood, we observed about 80% of classical monocytes, about 10% of intermediate monocytes and about 5% of non-classical monocytes (Figure 3). The stimulation of whole blood with either STB MV or exosomes from normal placentae induced a significant reduction of the percentage of classical monocytes (Figure 3). Moreover, both STB MV and exosomes increased the number of intermediate monocytes, with no effect on non-classical monocytes. Also, the percentage of granulocytes remained stable after stimulation with STB MV or exosomes from normal placentae compared to control (Figure 3).

![Graph showing percentage of monocyte subsets, granulocytes and activated cells after stimulation with normal syncytiotrophoblast extracellular vesicles](image)

Figure 3: Percentage of monocytes subsets, granulocytes and activated cells after stimulation with normal syncytiotrophoblast extracellular vesicles

Whole blood was stimulated for 24 h with normal syncytiotrophoblast microvesicles (STB MV) or exosomes and the percentage of classical monocytes (CM), intermediate monocytes (IM), non-classical monocytes (NCM) and granulocytes (G) was analyzed flow cytometric. Wilcoxon Signed Rank test: * p<0.05, ** p<0.01

**STB MV and exosomes from normal placentae increase the granularity of monocyte subpopulations and granulocytes.**

In control stimulation of whole blood, the monocyte subpopulations and granulocyte population formed a distinct population of cells (Figure 4). In contrast, the stimulation with either STB MV or exosomes from normal placentae induced a shift in the side scatter characteristics of classical and intermediate monocytes and granulocytes (Figure 4). The side scatter characteristics of the monocyte subpopulations and granulocytes following incubation with STB MV and exosomes is shown in Figure 5. STB EV from normal placentae significantly increased the side scatter of classical and intermediate monocytes and granulocytes. Additionally, the
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Stimulation with normal exosomes increased the side scatter of classical, intermediate and non-classical monocytes and granulocytes (Figure 5). While the side scatter was increased following stimulation with STB MV or exosomes, the forward scatter remained unaffected by the stimulation. Also, the side scatter of the granulocytes was significantly increased after stimulation with either STB MV or exosomes from normal placentae (Figure 5). With respect to the increase of the side scatter, the effect of exosomes from normal placentae was significantly stronger than the effect of STB MV from normal placentae. This increased granularity may suggest an increased activation of monocytes and granulocytes, which was tested next.

Figure 4: Forward (FSC) and sideward scatter (SSC) dot plots of classical, intermediate and non-classical monocytes and granulocytes after stimulation with normal syncytiotrophoblast extracellular vesicles.

Whole blood was stimulated for 24 h with normal syncytiotrophoblast microvesicles (STB MV) or exosomes and analyzed flow cytometric. The stimulation induced a shift of the populations of classical, intermediate and non-classical monocytes as well as granulocytes due to an increase of the side scatter (SSC).
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STB EV from normal placentae activate classical and intermediate monocytes and granulocytes.

In control stimulations, about 96% of classical monocytes, about 90% of intermediate and about 11% of non-classical monocytes were CD11b+ (Figure 6). The stimulation of whole blood with STB MV from normal placentae did not affect the percentage of CD11b positive monocytes. However, stimulation with exosomes led to a significant reduction of the percentage of CD11b+ intermediate monocytes compared to control and STB MV (Figure 6). The expression intensity of CD11b+ was increased on classical and intermediate monocytes after stimulation with both STB MV or exosomes from normal placentae while non-classical monocytes remained unaffected. Furthermore, stimulation of whole blood with STB MV and exosomes from normal placentae increased the percentage of CD11b+ granulocytes and the expression intensity of CD11b on granulocytes significantly (Figure 6). With respect to CD11b expression intensity, the effect of exosomes from normal placentae was significantly stronger than the effect of STB MV from normal placentae.

STB MV and exosomes from preeclamptic placentae affect numbers of monocyte subpopulations.

The stimulation of whole blood with exosomes from preeclamptic placentae induced a significant reduction of the percentage of classical monocytes (Figure 7). Also, the stimulation with either STB MV or exosomes from preeclamptic
placentae significantly increased the percentage of intermediate monocytes but did not have an effect on the percentage of non-classical monocytes. The percentage of granulocytes was also not affected by stimulation with either STB MV or exosomes from preeclamptic placentae (Figure 7).

**STB MV and exosomes from preeclamptic placentae increase the granularity of monocyte subpopulations and granulocytes.**

The stimulation of whole blood with either STB MV or exosomes from preeclamptic placentae significantly increased the side scatter of classical and intermediate monocytes (Figure 8), but the forward scatter remained unaffected. Also, the stimulation with exosomes from preeclamptic placentae significantly increased the side scatter of non-classical monocytes and granulocytes (Figure 8). With respect to the increase of the side scatter, the effect of exosomes from preeclamptic placentae was significantly stronger than the effect of STB MV from preeclamptic placentae.

**STB MV and exosomes from preeclamptic placentae activate monocytes.**

Although the percentage of CD11b+ classical and intermediate monocytes were not affected by either STB MV or exosomes from preeclamptic placentae, stimulation of whole blood with STB MV from preeclamptic placentae slightly, but significantly increased the percentage of CD11b+ non-classical monocyte (Figure 9). The CD11b expression intensity was significantly increased on classical monocytes after stimulation with STB MV and exosomes from preeclamptic placentae and on intermediate monocytes after stimulation with exosomes from preeclamptic placentae. No effect of STB MV or exosomes from preeclamptic placentae was seen on CD11b expression intensity on non-classical monocytes. Additionally, the stimulation with exosomes from preeclamptic placentae significantly increased the percentage of CD11b+ granulocytes as well as the CD11b expression intensity on those granulocytes (Figure 9). With respect to CD11b expression intensity, the effect of exosomes from preeclamptic placentae was significantly stronger than the effect of STB MV from preeclamptic placentae.
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**Figure 6:** Expression intensity of CD11b on monocytes and granulocytes after stimulation with normal syncytiotrophoblast extracellular vesicles

Whole blood was stimulated for 24 h with normal syncytiotrophoblast microvesicles (STB MV) or exosomes. The percentage of CD11b+ cells as well as the expression intensity of CD11b+ was analyzed by flow cytometry on gated classical monocytes (CM), intermediate monocytes (IM), non-classical monocytes (NCM) and granulocytes (G). Wilcoxon Signed Rank test: * p<0,05, ** p<0,01

**Figure 7:** Percentage of monocytes subsets, granulocytes and activated cells after stimulation with preeclamptic syncytiotrophoblast extracellular vesicles

Whole blood was stimulated for 24 h with preeclamptic syncytiotrophoblast microvesicles (STB MV) or exosomes and the percentage of classical monocytes (CM), intermediate monocytes (IM), non-classical monocytes (NCM) and granulocytes (G) was analyzed flow cytometric as. Wilcoxon Signed Rank test: * p<0,05, ** p<0,01
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**Figure 8:** Forward (FSC) and sideward scatter (SSC) of classical, intermediate and non-classical monocytes and granulocytes after stimulation with preeclamptic syncytiotrophoblast extracellular vesicles.

Whole blood was stimulated for 24 h with preeclamptic syncytiotrophoblast microvesicles (STB MV) or exosomes. The percentage of CD11b+ cells as well as the expression intensity of CD11b+ was analyzed by flow cytometry on gated classical monocytes (CM), intermediate monocytes (IM), non-classical monocytes (NCM) and granulocytes (G). Wilcoxon Signed Rank test: * p<0.05, ** p<0.01

**Figure 9:** Expression intensity of CD11b on monocytes and granulocytes after stimulation with preeclamptic syncytiotrophoblast extracellular vesicles.

Whole blood was stimulated for 24 h with preeclamptic syncytiotrophoblast microvesicles (STB MV) or exosomes and the FSC and SSC of classical monocytes (CM), intermediate monocytes (IM), non-classical monocytes (NCM) and granulocytes (G) were analyzed flow cytometric. Wilcoxon Signed Rank test: * p<0.05, ** p<0.01
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Discussion

Based on their different mode of formation and molecular load, it has been suggested that microvesicles and exosomes have different functions.[6] In the present paper, we studied the effects of placental microvesicles (STB MV) and exosomes on the activation of monocytes and granulocytes. To our knowledge, we are the first to directly compare the effects of STB MV and exosomes derived from the same placentae. We incubated whole blood samples of non-pregnant women with physiological levels of STB MV or exosomes in uncomplicated pregnancies and studied the effect of these vesicles on monocytes and granulocytes.

Our data show that both STB MV and exosomes from normal placentae decrease the number of classical monocytes and increase the number of intermediate monocytes in blood samples of non-pregnant women to a similar extend. As we incubated blood samples with the STB MV or exosomes, the increase in intermediate monocytes cannot be accounted for by recruitment from the bone marrow. Therefore, our data are in line with the suggestion that classical monocytes can mature into intermediate and possibly non-classical monocytes in the peripheral circulation[17]. We suggest that our recent finding of monocyte maturation from classical monocytes to intermediate monocytes during healthy pregnancy[12] may be, at least partly, due to STB MV and placental exosomes. The mechanism, however, by which the STB MV and exosomes affect monocyte maturation cannot be deduced from the present study and remains to be investigated. Nevertheless, other groups showed that STB MV may not only bind to monocytes but even be phagocytosed by monocytes, which is related to the induction of e.g. the expression of diverse cytokines, both type-1 and type-2 but with a dominance of pregnancy-favoring type-2 immunity.[18,19]

Not only monocyte maturation was affected, but also monocyte subpopulations were activated differentially by both normal STB MV or exosomes. We showed that STB MV increased the side scatter of both classical and intermediate monocytes. Increased side scatter characteristics indicate increased granulation of the cells, which is a sign of activation and differentiation of monocytes into macrophages.[20] Indeed, STB MV increased the expression of the activation marker CD11b on these subpopulations of monocytes. Also in vivo, increased expression of CD11b and other activation markers, such as CD14 and CD64, have been described on peripheral monocytes of pregnant women compared to non-pregnant women.[13,16] CD11b is known to be an important mediator of leucocyte adhesion, enabling leucocyte migration into specific tissues and in the case of monocytes also initiates differentiation into macrophages.[21] Monocytes have been described to refresh the pool of tissue macrophages by invading into specific tissues where they can differentiate into macrophages upon activation, with and without an intermediate differentiation step into dendritic cells.[17,22] During differentiation into macrophages, monocytes usually start expressing CD16 but lose their
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CD14 expression and it has been suggested, that predominantly CD14+CD16+ non-classical monocytes differentiate into macrophages.[17,20] However, a large percentage of decidual macrophages has been shown to be strongly positive for CD14 [23], suggesting that especially CD14++ monocytes are recruited to differentiate into decidual macrophages. Based on this hypothesis, the increased CD11b expression and granularity of CD14++ classical and intermediate monocytes in response to the stimulation with STB EV suggests an increased adhesion, migration and differentiation potential of classical and intermediate monocytes. In combination with the described data, our experiments indicate that the placenta may directly stimulate these monocyte subsets with STB EV to initiate differentiation into pregnancy-supportive anti-inflammatory decidual macrophages.

STB MV and exosomes from normal placentae also induced an increase of the side scatter and CD11b expression in granulocytes. It has been described by Sacks et al. that in healthy pregnancy, granulocytes are activated [13], characterized by, amongst others, increased CD11b expression [13,24]. Our results thus suggest, that STB MV and exosomes from normal placentae may also play a role in the granulocyte activation (like with monocytes) and thus, modulate granulocytes to support the tolerant state during pregnancy. The mechanism of activation remains obscure, however, a study by Lampé et al. suggests that neutrophils might be involved in the phagocytosis of STB EV and therefore in STB EV clearance from the maternal blood [25]. This could additionally lead to activation of the granulocytes. However, to our knowledge, a direct interaction of STB EV with granulocytes has not been shown yet. In a murine model, granulocytes have been shown to interact with lung-derived exosomes [26], indicating that an interaction with STB EV is highly possible.

Our data show that both STB MV and exosomes from normal placentae activate monocytes and granulocytes, however the effect of exosomes seems to be stronger. Unique markers for microvesicles and exosomes are existing [3,6], indicating that the molecular load of STB MV and exosomes might be partially unique to either fraction. This may explain the increased effects of exosomes. The ratio of STB MV over exosomes in healthy pregnancy is unknown at this moment. However, our own unpublished data (using NanoSight analysis) on STB MV and exosomes indicate that, at least from the perfused placenta, the ratio of secreted STB MV over exosomes is about 1. Therefore in our study, it is unlikely that differences in numbers between STB MV and exosomes can explain the different effects of these types of vesicles. Despite the fact that the molecular load of STB EV from preeclamptic placentae is different from that of STB EV from normal placentae [11], our data do not indicate a functional difference of STB EV from preeclamptic placentae versus STB EV from normal placentae. STB EV from preeclamptic placentae decreased the number of classical monocytes and increased the number of intermediate monocytes to the same extend as STB EV from healthy placentae. Moreover, exosomes from preeclamptic placentae induced an increase in granularity of all three monocyte subsets and granulocytes as well as increased CD11b expression on classical and interme
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diate monocytes and granulocytes, also to the same extend as exosomes from healthy placentae did. However, during PE, our group recently observed in vivo a decrease in classical monocytes and an increase in intermediate monocytes as compared to healthy pregnancy [12]. Moreover, others found an increased activation of monocytes in PE as compared with healthy pregnancy.[13,24] Therefore, our data suggest that during healthy pregnancy STB EV may induce the pregnancy-associated monocyte and granulocyte activation, but that during PE the STB EV are not involved in the further activation of monocytes and granulocytes. Other mechanisms may be involved. One of these mechanisms may be the increased number of STB EV in preeclamptic pregnancies versus normal pregnancies.[8,27] In our study, we compared the effects of equal amounts of normal and preeclamptic STB EV. The effects of different numbers of STB EV remain to be established. However, also the secretion of other factors (e.g. proteins like Soluble fms-like tyrosine kinase-1) may be involved in the induction of the exaggerated inflammatory response during PE.

In conclusion, we showed that STB MV and exosomes from normal placentae have an immune-regulatory function by inducing a monocyte subset shift from classical to intermediate monocytes and an activation of monocyte and granulocytes. Although it is assumed that microvesicles and exosomes might show different functions due to their divergent mode of formation and molecular load, STB MV and exosomes exhibited a similar effect on monocytes and granulocytes in our study. However, STB MV and exosomes from preeclamptic placentae were not able to induce the exaggerated phenotypical alterations in monocytes and granulocytes which have been observed during PE. Other factors must be responsible for this effect and need further research.
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References

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