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# Charakterisierung der Monozyten-Subpopulationen im Verlauf der gesunden humanen Schwangerschaft

**Dissertation**

zur Erlangung des Grades eines Doktors der Medizin
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# In-depth characterization of monocyte subsets during the course of healthy pregnancy

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ABSTRACT

Pregnancy represents an immunological challenge for the maternal immune system. Pregnancy augments innate immune responses, and particularly monocytes contribute to maintaining the balance between pro- and anti-inflammatory immune responses required for the successful sequence of distinct immunological phases throughout pregnancy. Nonetheless, studies that focus on the heterogeneity of monocytes and analyze the alteration of monocyte subsets in a longitudinal approach throughout healthy pregnancies have remained scarce.

In this study, we characterized the gradual phenotypic changes of monocyte subsets and the secretory potential of bulk monocytes in peripheral blood mononuclear cells of healthy pregnant women from a population-based prospective birth cohort study. Blood samples at predefined time points were analyzed using flow cytometry for in-depth characterization of monocyte subsets, which confirmed a shift from classical towards intermediate monocytes throughout pregnancy. Principal component analysis revealed characteristic phenotypic changes on monocyte subsets, especially on the intermediate monocyte subset, throughout pregnancy. Pregnancy-related hormones were measured in serum and β-human chorionic gonadotropin levels were significantly associated with expression of CD11b, CD116 and CCR2 on monocyte subsets. TLR4 and TLR7/8 stimulation of monocytes furthermore showed reduced polycytokine production towards the end of pregnancy.

These data provide a comprehensive overview of phenotypic changes and secretory potential of monocytes in healthy pregnant women and establish a selective contribution of different monocyte subsets to healthy pregnancy. The results from this study therefore build a basis for future comparisons and evaluation of women with adverse pregnancy outcomes.

## 1. Introduction

Pregnancy uniquely challenges the maternal immune system to establish immunological tolerance towards the semi-allogeneic fetus while simultaneously maintaining effective antimicrobial defense. Previous publications have elucidated that augmented innate immune responses, including monocytes as innate effector cells, contribute to this balance (Luppi et al., 2002b; Sacks et al., 1999). Innate immune activation is triggered by a combination of factors, including gestational hormones, leukocyte interaction with the placenta, soluble placental products, syncytiotrophoblast microparticles and fetal cells in the maternal circulation (Veenstra van Nieuwenhoven et al., 2003; Sacks et al., 1999).

Monocytes are grouped according to their surface expression of the pattern recognition receptor CD14 and the FcγIII receptor CD16 into classical, intermediate and non-classical subsets (Ziegler-Heitbrock

*Abbreviations:* BFA, Brefeldin A; CCL, C-C chemokine ligand; CCR, C-C chemokine receptor; CD, cluster of differentiation; FDR, false discovery rate; FSH, follicle-stimulating hormone; GM-CSF, granulocyte-monocyte colony-stimulating factor; hCG, human chorionic gonadotropin; LH, luteinizing hormone; MFI, median fluorescence intensity; PAMP, pathogen-associated molecular pattern; PRINCE, Prenatal Identification of Children's Health; TLR, Toll-like receptor; TSH, thyroid-stimulating hormone

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et al., 2010), which appear to be sequential developmental stages in monocyte differentiation (Patel et al., 2017; Wong et al., 2011) with distinct specializations. Classical monocytes are capable phagocytes, preferentially respond to stimuli like pathogens or inflammation and are involved in chemotaxis and tissue repair. Intermediate monocytes particularly contribute to antigen presentation, apoptosis regulation and cytokine secretion, while non-classical monocytes are highly differentiated, patrol the endothelium and express genes required for Fc receptor-mediated phagocytosis (Gren et al., 2015; Wong et al., 2011; Hofer et al., 2015).

Monocyte activation throughout pregnancy induces profound changes in subset composition, phenotype and function. Monocytes as professional phagocytes in the bloodstream are capable to engulf syncytiotrophoblast microparticles and trophoblast-derived exosomes resulting in monocyte activation (Southcombe et al., 2011; Messerli et al., 2010; Atay et al., 2011). The uteroplacental passage has also been reported to induce monocyte activation during pregnancy (Mellembakken et al., 2002). High concentrations of 17β-estradiol appear to exert anti-inflammatory effects on monocytes, but the influence of sex hormones on monocyte activation *in vitro* is contradictory (Bouman et al., 2005). Monocyte activation can be monitored by soluble markers in the blood like sCD14 and sCD163, which are shed in response to pro-inflammatory stimuli (Chenevier-Gobeaux et al., 2015; Zhi et al., 2018), or CC-chemokine ligand 2 (CCL2), a chemokine mainly secreted by monocytes and macrophages (Deshmane et al., 2009). Studies focusing on monocytes during healthy pregnancy univocally describe their activation, but the results on concomitant phenotypic alterations and cytokine production diverge (Faas and de Vos, 2016). Possible reasons might be the timepoint of sampling, cross-sectional study design or methodical aspects. Dysregulated, further augmented monocyte activation has been demonstrated in preeclampsia (Naccasha et al., 2001; Al-ofi et al., 2012; Faas and de Vos, 2016; Mellembakken et al., 2002), a pregnancy disorder considered to result from impaired trophoblast implantation. Since monocytes contribute to successful trophoblast implantation and remodelling, serve as a first line of defence for pathogens entering the blood stream and extravasate into inflamed tissue (Faas et al., 2014; Veenstra van Nieuwenhoven et al., 2003), they represent a relevant field of research in pregnancy.

Pregnancy is characterized by distinct immunological phases with respectively adapted cytokine milieus, which allow for the varying processes of implantation, placentation, growth and parturition (Mor and Cardenas, 2010). Several studies have investigated the role of the immune system in adverse pregnancy outcomes, but longitudinal studies concentrating on the biology of healthy pregnancies throughout all immunological phases are scarce. Especially studies focusing on the heterogeneity of monocyte subsets throughout pregnancy are lacking, although the phenotypic and functional characteristics of overall monocytes change significantly. Considering this, blood samples from healthy pregnant women were collected prospectively at defined time points in each trimester. Sex hormone levels, monocyte activation status, in-depth phenotypic characteristics of monocyte subsets and monocyte secretory potential were analyzed. Surface markers for phenotypic analysis were selected based on reported differential expression either on monocyte subsets or during pregnancy on bulk monocytes, in order to investigate functionally important markers and to transfer previous findings to monocyte subsets. Those included adhesive and chemotactic markers like CD11b and CCR2, markers involved in activation and differentiation like CD64, CD116 and CD132 and immunoregulatory markers like CD172a/b.

This study thus aimed at a thorough assessment of the phenotypic and functional changes of monocyte subsets upon activation during the course of pregnancy to evaluate their possible contribution to immunological tolerance.

## 2. Materials and methods

### 2.1. Study design

Pregnant women were recruited to the PRINCE (Prenatal Identification of Children's Health) study, a population-based prospective birth cohort study in Hamburg, Germany. Non-smoking women of adult age with singleton pregnancy and natural conception were enrolled at the University Medical Center Hamburg-Eppendorf (UKE). At the end of each trimester (gestational week 13–15, 23–25 and 35–37) study participants presented for detailed fetal and maternal examination with collection of maternal blood. For activation status, hormonal measurements and phenotypic analysis frozen serum samples and frozen peripheral blood mononuclear cells (PBMCs) from 15 participants were analyzed retrospectively (Table 1). Cryopreserved samples were chosen to establish the best intra- and inter-individual comparability, by minimizing changes due to technical variation in the measurements over time. Exclusion criteria for the investigated subgroup were pregnancy complications, immunosuppressive medication and chronic or gestational infectious diseases. For the assessment of monocyte function fresh blood samples from another subgroup of 21 participants of the PRINCE cohort were utilized (supplemental table 3 and elsewhere (Ziegler et al., 2018)). Non-pregnant female and male individuals were cross-sectionally enrolled at the UKE for a single blood collection and served as age-matched controls. Serum tubes were processed as indicated by the manufacturer. EDTA blood was used for isolation of PBMCs by Biocoll (Biochrome/Merck) gradient centrifugation. Samples were cryopreserved until conduction of phenotypic and hormonal assessment. For detailed protocols, please refer to the supplemental. The ethical commission of the Ärztekammer Hamburg authorized the studies (PV3694, PV4780) and each participant gave written informed consent prior to enrolment.

### 2.2. Measurement of pregnancy-associated hormones in serum

Serum concentrations of female reproductive hormones were quantified at the Central Laboratory of the UKE. 17β-estradiol and progesterone levels were assessed by chemiluminescence-immunoassay on ADVIA Centaur XP systems (Siemens Healthineers). Total β-human chorionic gonadotropin (β-hCG) (the intact hCG heterodimer and the free β subunit) levels were assessed by a homogenous LOCI® chemiluminescent sandwich immunoassay using Dimension Vista 1500 analyzers (Siemens Healthineers). All methods adhered to the manufacturer's protocols and the standard operation procedures of the laboratory. The

**Table 1**
**Participant characteristics.** 15 healthy, pregnant women with natural conception and singleton pregnancies were enrolled. 15 age-matched, healthy non-pregnant women and men were recruited as controls. Age is depicted as median [interquartile range]. Weight and gravidity are depicted as percentage value with absolute frequencies.

| PRINCE | characteristics | total $n$ = 15 |
|---|---|---|
| *Age (years)* | 29 [24 – 34] | |
| *Weight* | Mean BMI ± 1SD (kg/m$^2$) | 23.87 ( ± 3.5) |
| | BMI = 18,5 – < 25 (kg/m$^2$) | 73.3 % ($n$ = 11) |
| | BMI = 25 – < 30 (kg/m$^2$) | 13.3 % ($n$ = 2) |
| | BMI ≥ 30 (kg/m$^2$) | 13.3 % ($n$ = 2) |
| *Gravidity* | Primigravida | 66.7 % ($n$ = 10) |
| | Multigravida | 33.3 % ($n$ = 5) |
| controls | characteristics | total $n$ = 15 |
| *Age (years)* | | |
| | female | 29 [26 – 34] |
| | male | 28 [24 – 33] |

three serum samples from one pregnant woman were excluded from the analysis due to technical issues.

### 2.3. Phenotypic analysis of monocyte subsets

PBMCs were thawed and subsequently stained and analyzed using multiparameter flow cytometry. Flow cytometric data were acquired on a BD LSRFortessa (BD Biosciences). On each experimental run, the samples of one pregnant woman from each trimester were assessed together with one age-matched female and male control. Three distinct flow cytometric panels were implemented for immunophenotyping. All antibodies were titrated and used at optimal staining concentrations (supplemental Table 1). For each panel, an individual set-up of the flow cytometer (PMT-voltages and compensation) was optimized and tested for reproducibility. Surface marker expression was compared between fresh and cryopreserved monocytes in advance (supplemental figure 5). Measurements were performed within three weeks using the same flow cytometer with identical, daily adapted measurement pre-adjustments with one exception (one experimental run was acquired at another LSRFortessa, using the same configuration).

### 2.4. Assessment of monocyte secretory function

The secretory potential of monocytes was quantified by the assessment of intracellular TNFα, IL-12 and IL-6 at baseline and upon lipopolysaccharide (LPS) and CL097 stimulation as previously described (Ziegler et al., 2018). In brief, PBMCs were isolated from EDTA blood samples latest 3 h after venepuncture using density gradient centrifugation. PBMCs were maintained in RPMI-1640 (Life Technologies) supplemented with 10 % heat-inactivated FBS (Biochrome AG). PBMCs were stimulated with 1 µg/ mL CL097 (Invivogen) or 100 ng/ mL LPS (Sigma) or left unstimulated, each in the presence of 5 µg/ mL Brefeldin A (Sigma) for 17 h.

## 3. Data analysis

Flow cytometric data were analyzed using FlowJo software v10.4.2 (FlowJo, LLC). Hierarchical and Boolean gating was performed to identify specific subsets. For graphical display of data, FlowJo software v10.4.2 and GraphPad Prism 8.2.0 (GraphPad Software, Inc.) were used.

Statistical analyses of the data were conducted using GraphPad Prism 7.04. Wilcoxon signed rank tests were applied for paired comparison of the pregnant women (pairwise comparisons of time points within pregnant women) and Mann-Whitney *U* test to determine statistical significance between the groups (non-pregnant controls versus pregnant women at a given time point). Test multiplicity for all tests was adjusted using the false discovery rate (FDR) method of Benjamini and Hochberg (Benjamini and Hochberg, 1995). All reported p-values are FDR-adjusted, $p < 0.05$ are significant.

Principal component analysis was done using XLSTAT (version 2018.7.55140, Addinsoft, Paris, France) and included flow cytometric data acquired at the same BD LSRFortessa only (n = 14). For the heat map, the median standardized values of each surface marker were calculated and plotted in the heat maps for the three different monocyte subsets for all analyzed groups, respectively. To standardize the median fluorescence intensity (MFI), the mean MFI of all individuals and subsets was subtracted from the individual MFI and the difference was divided by the standard deviation (SD): Y* = (Y-mean MFI) / SD.

## 4. Results

### 4.1. Participants

The PRINCE cohort of healthy pregnant women collects blood samples at predefined time points during pregnancy and thus offers a valuable collection to study significant changes in immune cell phenotypes over the course of pregnancy. From each woman one sample per trimester was analyzed. Age-matched, non-pregnant women and men served as controls. The detailed participant characteristics for the phenotypic examination of monocyte subsets are provided in Table 1.

### 4.2. Composition of circulating monocytes throughout pregnancy

We analyzed and compared the composition and the phenotype of monocyte subsets trimester-wise in PBMCs from pregnant women using flow cytometry. After exclusion of dead cells, monocytes were defined as $lin^{neg}$, $HLA\text{-}DR^{pos}$ and $CD14^{pos}$. Of note, $CD56^{pos}$ monocytes were analyzed separately (data not shown). Their mean proportion of live PBMCs ranged from 0,13 − 0,17 % throughout pregnancy and in the non-pregnant controls. The three monocyte subsets were distinguished by their surface expression of CD14 and CD16 molecules as following: $CD14^{high}$ $CD16^{neg}$ (classical), $CD14^{high}$ $CD16^{pos}$ (intermediate) and $CD14^{pos}$ $CD16^{high}$ (non-classical) monocytes (Fig. 1A). The proportion of overall $CD14^{pos}$ monocytes among live PBMCs increased towards the end of pregnancy (Fig. 1B). We confirmed observations from previous studies (Ziegler et al., 2018; Groen et al., 2015; Melgert et al., 2012) that there is a significant shift in the composition of monocytes from classical towards intermediate monocytes (Fig. 1C and D), while there was no difference in the percentage of non-classical monocytes (Fig. 1E). In addition, pregnant women had significantly fewer classical monocytes (Fig. 1C) and significantly more intermediate monocytes (Fig. 1D) in comparison to non-pregnant women, while there was no significant difference in the amount of non-classical monocytes between pregnant and non-pregnant women (Fig. 1E). In sum, the increase of overall monocytes throughout pregnancy is related to a rising proportion of the intermediate subset, whereas the classical subset becomes diminished.

### 4.3. Monocytes from pregnant women can be distinguished from monocytes from non-pregnant women based on their phenotype

To assess the activation state of monocytes during pregnancy, soluble markers reflecting monocyte function were quantified in serum of pregnant women. During the course of pregnancy, the levels of sCD163 and sCD14 significantly increased (supplemental Fig. 1). In contrast, CCL2 levels significantly decreased (supplemental Fig. 1).

To study monocyte subsets' activation in detail, we developed flow cytometric measurements and selected markers based on reported differential expression either during pregnancy or on monocyte subsets. A comprehensive overview of the selected markers can be found in supplemental table 2. Surface expression of three markers (CD69, CD73, CD274) was not detectable on frozen monocytes after thawing and consequently excluded from further analysis. Principal component analysis revealed that non-pregnant and pregnant women diverged substantially based on the expression levels of surface antigens on their respective monocyte subsets (Fig. 2A). Those differences remained constant throughout gestation and altered hormone levels (data not shown). Monocytes from pregnant women did not group by trimester of pregnancy, but rather by individual women (supplemental Fig. 2). Furthermore, intermediate monocytes separated from non-classical monocytes, with intermediate monocytes being more closely related to pregnancy throughout all trimesters (Fig. 2B, showing $3^{rd}$ trimester only). Especially the expression of CD132, CD14, CD64, CD11b and CD116 on intermediate monocytes, CD32 on classical monocytes and TLR4 expression on classical and non-classical monocytes was associated with pregnancy (Fig. 2B, upper right quadrant).

Fig. 2C summarizes the expression levels of surface antigens on the three different monocyte subsets throughout the course of pregnancy. Of note, intermediate monocytes (Fig. 2C, middle column) expressed the highest amount of most of the markers of interest and underwent strong phenotypic changes throughout the course of pregnancy.

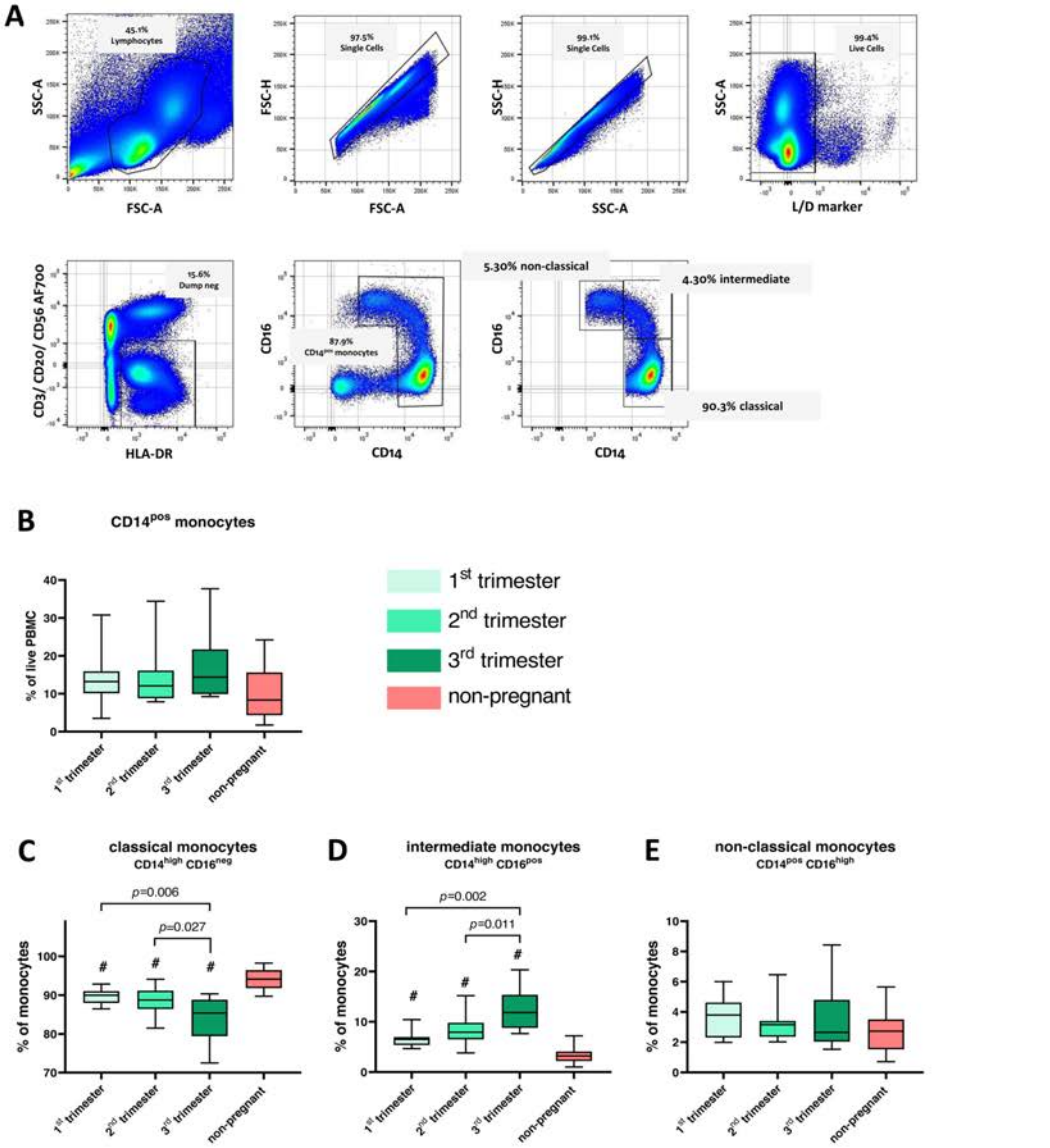


**Fig. 1. Monocyte subset composition changes during the course of pregnancy.** PBMCs of pregnant women (n = 15) were surface stained to allow for phenotypic identification of monocyte subsets. (A) Representative dot plot of flow data, comparison of (B) bulk $CD14^{pos}$ monocytes, (C) $CD14^{high}CD16^{neg}$ classical monocytes, (D) $CD14^{high}CD16^{pos}$ intermediate monocytes, (E) $CD14^{pos}CD16^{high}$ non-classical monocytes (box-and-whisker plot, whiskers: min to max; Mann-Whitney *U* test and Wilcoxon signed rank test, FDR-adjusted p-values are displayed; # = significant difference compared to non-pregnant females).

Generally, monocyte subsets highly differ in the expression of surface markers involved in activation and differentiation (supplemental Fig. 3). The expression levels of CD11b, CD39, CD54, CD64 and CD116 on all CD14 positive monocytes (see gating strategy) were significantly elevated in the third trimester compared to the first trimester (supplemental Fig. 4), with a trend towards gradually increasing MFIs of CD11b, CD39, CD54 and CD64 throughout pregnancy. Detailed phenotypic analysis of monocyte subsets revealed the following changes: CD54 expression was increased only on the classical monocyte subset during the course of pregnancy. CD14, CD132, CD172a/b and CCR2 (C-C chemokine receptor type 2) progressively increased on intermediate monocytes during the course of pregnancy. CD11b, CD64 and CD116 increased on the classical and intermediate subset. We were not able to detect any significant changes in the phenotype of the non-classical monocyte subset (Fig. 2D, supplemental table 4). In summary, monocyte subsets undergo detailed and specific changes throughout pregnancy which enables them to be distinguished from non-pregnant females. Taken together, these data illustrate that pregnancy-associated

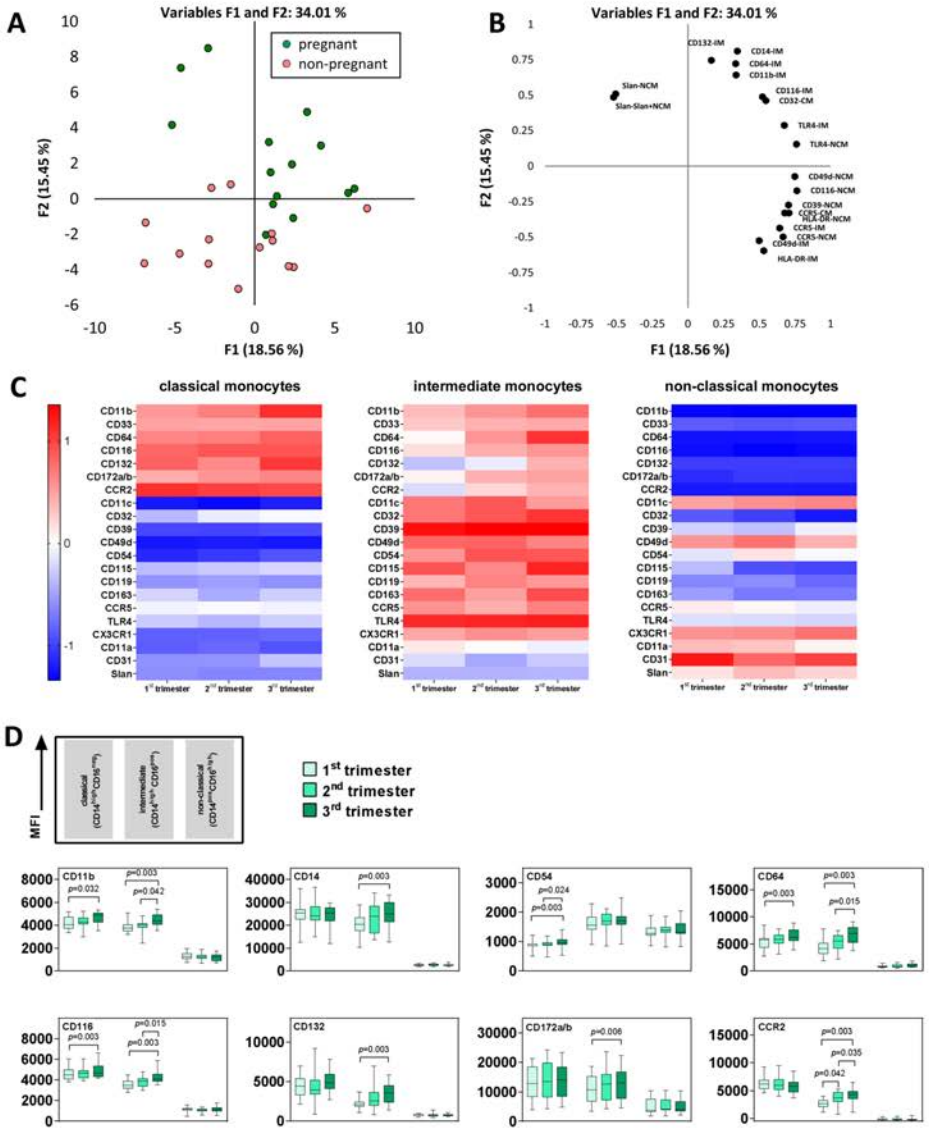


**Fig. 2. Monocyte subsets from pregnant women have a unique phenotype throughout pregnancy.** PBMCs from pregnant women (n = 15) were surface stained and analyzed for their phenotype of monocyte subsets throughout the course of pregnancy. (A + B) Principal component analysis (PCA) of phenotypic monocyte data of non-pregnant and 3rd trimester pregnant women. (A) Projection of participants on PCA 1st and 2nd components. (B) Biplot of the variables. All phenotypic data were included in the calculation of the components but only variables with good representation (i.e. sum(cos2) > 0.5) are shown on the plot. (C) Heat map with median standardized values. The median fluorescence intensity (MFI) of each subset in all individuals was standardized by subtraction of the mean MFI of all individuals and subsets and division of the difference by the standard deviation (SD): Y* = (Y-mean MFI) / SD. (D) Median fluorescence intensities of surface antigens on monocyte subsets were compared between the three trimesters of pregnancy (box-and-whisker plot, whiskers: min to max; Wilcoxon matched-pairs signed rank tests, FDR-adjusted p-values are displayed, see supplemental table 4).

activation of monocytes is reflected by alteration of surface markers involved in activation and differentiation, primarily in the intermediate monocyte subset.

### *4.4. B-hCG is associated with subset-specific surface marker expression at defined stages of pregnancy*

Pregnancy-related hormones can support the transition from inflammatory towards anti-inflammatory responses. We determined the serum levels of 17β-estradiol, progesterone and β-hCG throughout pregnancy (Fig. 3A), and correlated surface markers of monocyte subsets that were significantly changed throughout pregnancy (Fig. 2D) to pregnancy-related hormone levels. B-hCG was associated to the phenotype of monocyte subsets, but estradiol and progesterone were not (Fig. 3B, supplemental table 5). In detail, there was a significant negative association of β-hCG concentrations with (i) the expression levels of CD116 on classical monocytes in the first trimester of pregnancy, (ii) a significant negative association with the expression levels of CD11b

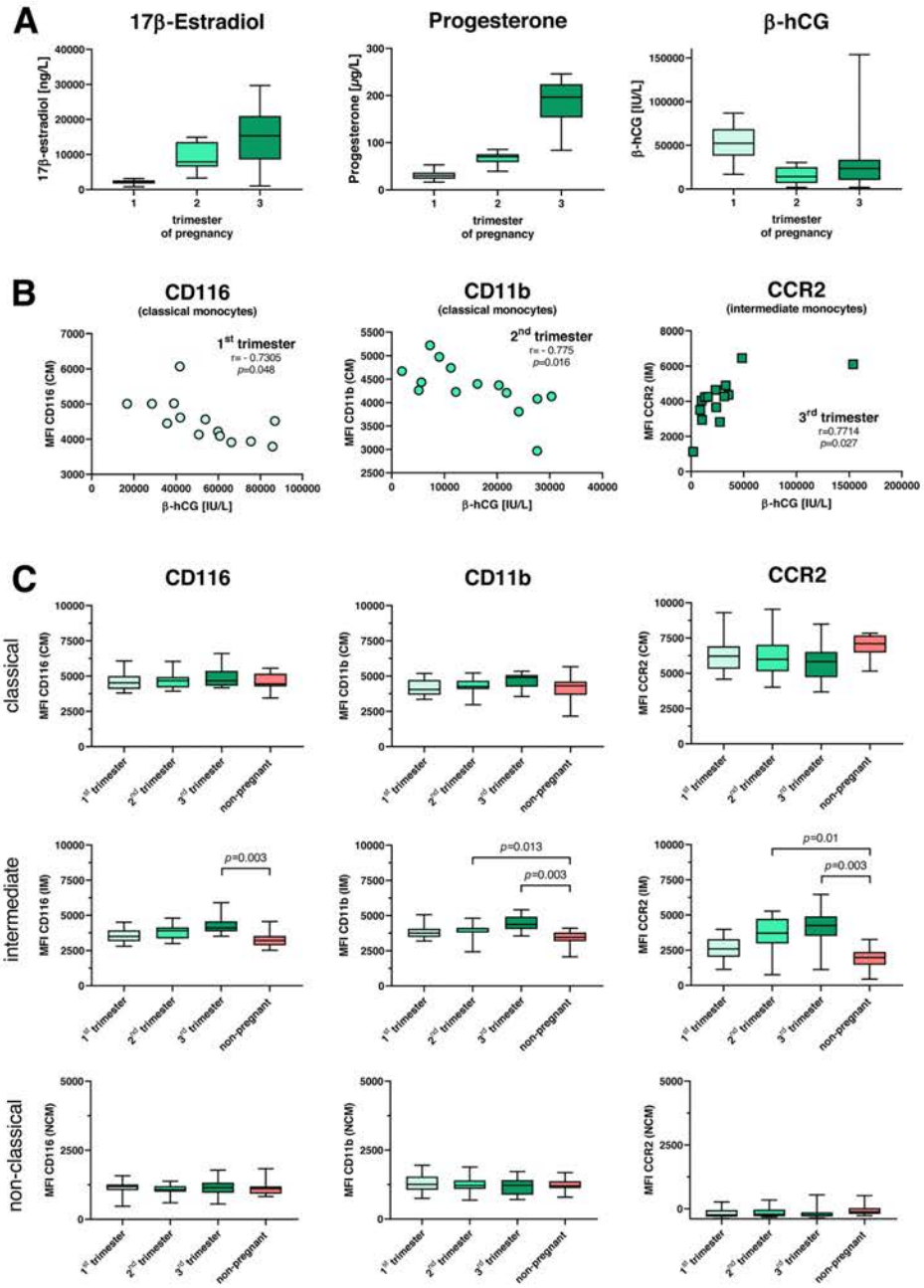


**Fig. 3. Pregnancy-associated hormones are associated with the phenotype of monocyte subsets.** (A) Serum levels of 17β-estradiol, progesterone and β-hCG were measured at three time points during pregnancy, representing 1st, 2nd and 3rd trimester of pregnancy. Serum samples from one participant were excluded from the analysis due to technical issues ($n$ = 14; box-and-whisker plot, whiskers: min to max, light green dots = 1st trimester; medium green dots = 2nd trimester, dark green dots = 3rd trimester). (B) Association of CD116, CD11b and CCR2 expression of monocyte subsets with β-hCG serum levels (spearman correlation, FDR-adjusted p-values are displayed, see supplemental table 5). (C) Monocyte subsets from pregnant women were analyzed for their median fluorescence intensity of CD116, CD11b and CCR2 and compared to non-pregnant women (n = 15; box-and-whisker plot, whiskers: min to max; Mann-Whitney U test, FDR-adjusted p-values are displayed). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

on classical monocytes in the second trimester of pregnancy and (iii) a significant positive association of the expression levels of CCR2 on intermediate monocytes in the third trimester of pregnancy (Fig. 3B). To furthermore ascertain the association of the changes in expression levels of CD116, CD11b and CCR2 to pregnancy, we compared the expression levels of the mentioned markers on monocyte subsets from

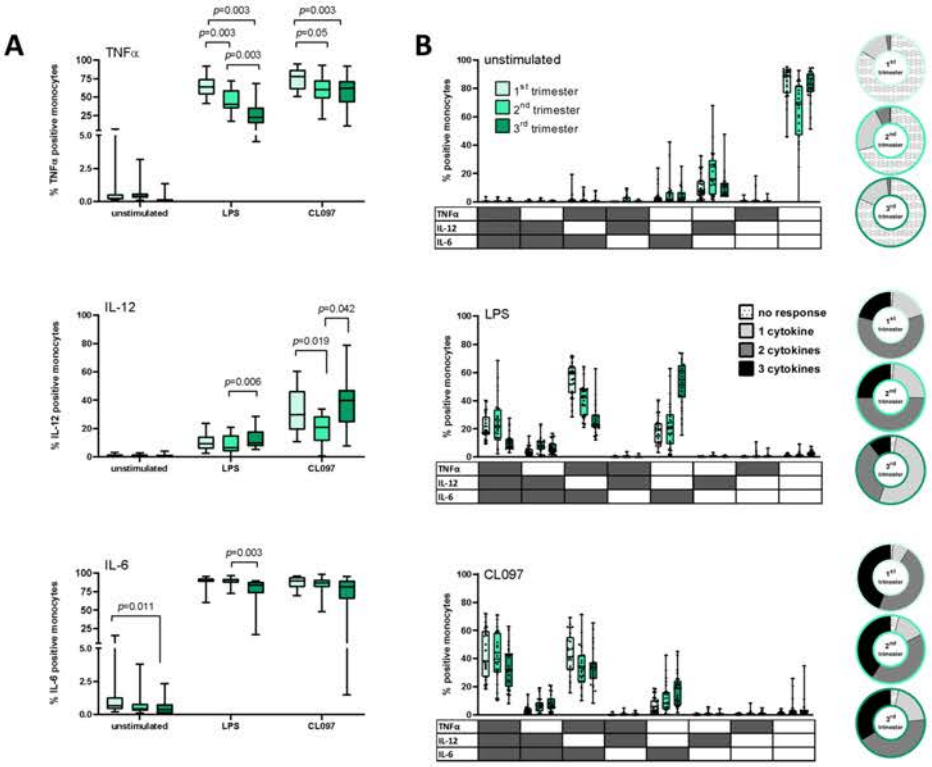


**Fig. 4. LPS- and CL097-induced cytokine production by monocytes during the course of pregnancy.** PBMCs from pregnant women (n = 21) were *ex vivo* stimulated with LPS or CL097 or left unstimulated in the presence of BFA. After 17 h intracellular staining for TNFα, IL-6 and IL-12 was performed. (A) Percentage of cytokine positive monocytes after 17 h is shown (box-and-whisker plot, whiskers: min to max; Mann-Whitney *U* test; FDR-adjusted p-values are displayed). (B) Polysecretory potential of PBMCs was determined using Boolean gating (FlowJo; box-and-whisker plot, all values). Tables below graphs depict the combination of cytokines. Pie charts summarize the distribution of polysecretory potential across the three trimesters..

pregnant women to non-pregnant women. There was a significant difference in expression levels of CD116, CD11b and CCR2 on intermediate monocytes between non-pregnant and pregnant females in the third (CD116) or in both the second and the third trimester of pregnancy (CD11b, CCR2) (Fig. 3C). Overall, these data show that changes in the phenotype of monocyte subsets during the course of pregnancy are associated with levels of β-hCG.

### *4.5. Intracellular production of IL-6, IL-12 and TNFα in monocytes shifted towards single-cytokine production during the course of pregnancy*

To investigate potential impacts of monocytes on inflammatory responses in pregnant women, we studied the secretory potential of circulating monocytes. We stimulated PBMCs, freshly isolated from pregnant women at the end of each trimester, with LPS (TLR4 ligand) or CL097 (TLR7/8 ligand). The proportions of cells producing TNFα, IL-12 or IL-6 were quantified using intracellular cytokine staining and subsequent flow cytometry.

In comparison to pregnant women from the third trimester, monocytes from first trimester pregnant women had higher basal levels of IL-6 (Fig. 4A). Furthermore, there was a significant drop in the proportion of $TNF\alpha^{pos}$ monocytes after both LPS (Ziegler et al., 2018) and CL097 stimulation, and in the proportion of $IL\text{-}6^{pos}$ monocytes after LPS stimulation towards the end of pregnancy (Fig. 4A). In contrast, the proportion of $IL\text{-}12^{pos}$ monocytes after LPS and CL097 stimulation decreased in the second trimester to significantly increase again in the third trimester of pregnancy (Fig. 4A). Finally, we assessed the polycytokine production of stimulated monocytes based on TNFα, IL-12 and IL-6 production across the three trimesters using Boolean gating. Overall, the production of multiple cytokines in one monocyte after stimulation decreased towards the end of pregnancy, whereas monocytes not responding to stimulation and single cytokine-producing monocytes increased throughout pregnancy (Fig. 4B, pie charts). IL-6, but not TNFα and IL-12–producing monocytes seemed to gain importance in the third trimester of pregnancy (Fig. 4B). In sum, the secretory potential of monocytes responding to stimulation is altered during healthy human pregnancy, with a specific reduction of polycytokine responses.

## 5. Discussion

Monocytes contribute to maintaining the balance between pro- and anti-inflammatory immune responses throughout pregnancy. In this study, phenotypic analysis of monocyte subset alteration during healthy pregnancy revealed that the shift in monocyte composition is accompanied by significantly upregulated surface expression of markers involved in differentiation, adhesion and activation upon stimulation with PAMPs, humoral and cellular signals. The upregulation was most pronounced in the intermediate monocyte subset and could in part be associated to serum levels of β-hCG. Functionally, we detected an increased monocytic IL-12 production in the third trimester upon stimulation, whereas baseline and stimulated TNFα and IL-6 production as well as polysecretory potential in monocytes progressively decreased.

It is hypothesized that intermediate and non-classical monocytes arise from classical monocytes (Patel et al., 2017) and gradually proceed in their stage of maturity (Ancuta et al., 2009). During pregnancy, the proportion of classical monocytes significantly shrinks in favor of a rising fraction of intermediate monocytes (Melgert et al., 2012; Groen et al., 2015; Ziegler et al., 2018). Similarly, infectious diseases with the human immunodeficiency virus type 1 (HIV-1), Zika virus and congenital toxoplasmosis as well as (auto-) inflammatory conditions were shown to increase the intermediate monocyte subset (Wacleche et al., 2018; Michlmayr et al., 2017; Machado et al., 2014), suggesting a proinflammatory surrounding to promote the expansion of intermediate monocytes. During pregnancy, it has furthermore been proposed that the classical monocyte subset is diminished due to recruitment to the feto-maternal interface, with augmented activation of the remaining monocytes to preserve effective protection against infections (Svensson-Arvelund et al., 2013). In this context, the intermediate monocyte subset has been suggested to maintain immunoregulatory functions by interacting with regulatory T cells (Faas and de Vos, 2016). Thus, the increased proportion of intermediate monocytes might contribute to precise adjustment of immunological responses throughout pregnancy.

The detailed phenotypic characterization of the three monocyte subsets in this study identified several markers on monocytes that exhibit strong changes under the influence of pregnancy, especially reflected by their association with the glycoprotein hormone β-hCG and their differential expression between pregnant and non-pregnant women. Among those were CCR2, CD11b and CD116. Interestingly, the surface expression of these markers during the first trimester of pregnancy did not significantly differ from non-pregnant women. However, among pregnant women of the first trimester, where β-hCG levels are peaking, surface expression of CD116 on classical monocytes was significantly negatively associated with β-hCG concentrations. CD116 (GM-CSFRα), despite its pivotal role in monocyte differentiation and function, has not been well characterized on monocytes of healthy pregnant women. Here, we identified an increase in CD116 expression on classical and intermediate monocytes towards the end of pregnancy. This increase together with the absence of changes during the first trimester and association to β-hCG levels reflects the immunological phases of pregnancy. The first trimester is considered an inflammatory state (Mor and Cardenas, 2010) and may require reduction of monocyte differentiation and effector functions either to reduce regulatory immune responses or to avoid exaggerated inflammatory responses. Considering the proinflammatory secretory potential during first trimester, the latter appears more likely. CD11b has often been examined in pregnancy with contradictory results (Sacks et al., 1998; Luppi et al., 2002a; Mikhaylova et al., 2013; Luppi et al., 2002b). It is presumed that the most remarkable up-regulation of this adhesive surface marker is associated with the onset of labor, indicating further monocyte activation (Luppi et al., 2002a). In contrast, others could not detect any significant differences in CD11b associated with pregnancy (Zhang et al., 2017). Similar to CD116, we identified an increase in CD11b on classical and intermediate monocytes towards the end of pregnancy and a strong negative association with β-hCG in the 2$^{nd}$ trimester. Taking the upregulation of further activation, differentiation and adhesive markers into account, increased monocyte evasion and subsequent differentiation into monocyte-derived dendritic cells and macrophages with the progression of pregnancy appears to be likely. This process may be supported by a progressive increase of GM-CSF, the ligand to CD116, throughout pregnancy (Vassiliadis et al., 1998). Again, a well-adjusted balance seems to be vital, since raised amounts of GM-CSF as well as macrophages have been found in preeclamptic decidua (Huang et al., 2010) and augmented CD11b expression on monocytes has been reported in preeclamptic pregnancies (Faas and de Vos, 2016).

CCR2 recruits monocytes to sites of inflammation. Published data on the expression of CCR2 on monocytes during pregnancy either show a decrease in receptor expression (Mikhaylova et al., 2013; Al-ofi et al., 2012), or a significant increase during term labor as compared to first and second trimester of pregnancy (Zhang et al., 2017). Its ligand, CCL2, is highly transcribed by human decidual cells and progesterone and high levels of β-hCG were shown to increase CCL2 secretion (He et al., 2007; Caballero-Campo et al., 2002). We detected a progressive decrease in CCL2 levels in serum samples of third trimester pregnant women accompanied by significantly increased expression of CCR2 on intermediate monocytes, but not on other monocyte subsets. CCR2 expression was furthermore positively associated with the concentration of β-hCG in participants' sera in the third trimester. B-hCG is able to interact with LH, FSH and TSH receptors, but the expression of those receptors on peripheral immune cells is controversial (Robinson et al., 2010). Non-canonical receptors for β-hCG on immune cells have been suggested, including TLR4 (Zamorina and Shirshev, 2014), C-type lectins (Kosaka et al., 2002), or the mannose receptor CD206 (Kane et al., 2009). *In vitro* exposure of PBMCs or THP-1 cells to different concentrations of β-hCG did not reveal any phenotypic changes within the monocyte population, suggesting no direct effect of β-hCG on monocytes (data not shown). Further studies are needed to test if β-hCG, acting on human decidual cells, results in the attraction of CCR2-expressing intermediate monocytes at different stages of pregnancy, potentially by increasing the expression of CCL2 in the decidua which creates an elevated chemokine gradient.

We furthermore analyzed the secretory potential upon TLR-stimulation of monocytes during pregnancy. Functional changes in monocytes from pregnant women have been studied with inconsistent results (Faas and de Vos, 2016). It is mainly assumed that monocyte activation during pregnancy results in increased tolerance towards LPS (Faas et al., 2002), causing decreased cytokine responses. Consistently, we observed diminished IL-6 and TNFα production upon LPS stimulation towards the end of pregnancy, although the intermediate monocyte subset with a proinflammatory function increases during pregnancy. Both elevated IL-6 and TNFα levels in the amniotic or vaginal fluid have been described to predict preterm birth (Yockey and Iwasaki, 2018), thus requiring tight regulation towards the end of healthy pregnancy. The dampened IL-12 production solely in the second trimester parallels the natural course of pregnancy immunology, with a pro-inflammatory state in the first trimester and around parturition opposed to an anti-inflammatory milieu in the second trimester during fetal growth (Mor and Cardenas, 2010). TLR7 and -8 participate in early innate immune responses to relevant gestational infections with ssRNA viruses like influenza virus, Zika virus or rubella virus, which may have strong negative impacts on the normal course of pregnancy for mother and child (Racicot and Mor, 2017; Goldenberg et al., 2000). The enhanced IL-12 production upon TLR7/8 stimulation in the third trimester of pregnancy presumably reflects augmented antiviral capacity, since IL-12 effectively promotes Th1 differentiation and effector functions (Ramshaw et al., 1997). Furthermore, our data revealed a possible role for monocytes with specific functions becoming more important in the last trimester of pregnancy. Overall, monocytes show a decrease in their polysecretory potential towards the end of pregnancy. Given that proinflammatory signalling contributes to the induction of parturition, and especially increasing IL-6 levels seem to play a crucial role (Peltier, 2003), the increased share of monocytes producing IL-6 only upon LPS stimulation in the third trimester might support this process.

Healthy pregnancies resulting in healthy infants have not been a major focus of research. Studies on the maternal immune system during physiological pregnancies will help to understand pathophysiological reactions during gestation and provide the basis for linking subsequent adverse outcomes of mother and child to the maternal immune system during pregnancy. Our work is the first study to describe detailed phenotypic changes on selected monocyte subsets in each trimester and to associate these findings to the level of β-hCG in healthy pregnant women. In addition, the specific adaptations of monocyte secretory potential to pregnancy were elucidated. This study thus provides important reference data on the phenotype and function of monocyte subsets at each state of healthy human pregnancy, enabling future

comparison to pregnancies with adverse outcomes, including pre-eclampsia.

## Authorship

S.M.Z. and M.A. designed the study. C.P., C.N.F., S.H.H. and S.M.Z. conducted the experiments. J.G., A.D., K.H., and P.C.A coordinated the PRINCE cohort of pregnant women and provided the participant samples. L.R. performed and gave advice on statistical analysis. V.J. and T.R. performed the hormone measurements.

## Disclosures

No disclosures.

## Declaration of Competing Interest

None.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jri.2020.103151.

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## Appendix A. Supplementary data

**Methods used for cryopreservation.**
Serum tubes were processed as indicated by the manufacturer. In brief, freshly filled serum tubes were rested for 30 minutes in an upright position. Afterwards, the serum tubes were centrifuged for 10 minutes at 2000xg at room temperature.
PBMCs obtained from the PRINCE cohort were resuspended in a solution of 90% RPMI and 10% FBS and then gradually diluted with a solution of 40% RPMI, 40% FBS and 20% DMSO in the laboratory of the department of Obstetrics and Fetal Medicine, University Medical Center Hamburg-Eppendorf. PBMCs obtained from the controls were directly resuspended and diluted in a solution of 90% FBS and 10% DMSO in the Heinrich Pette Institute, Leibniz Institute for Experimental Virology. Cryotubes were put in a precooled freezing container, transferred to the -80°C freezer for cooldown and stored in liquid nitrogen.

**Methods used for phenotypic analysis.**
PBMCs in cryotubes were pre-thawed in the water bath (37°C), slowly resuspended in prewarmed R10 and centrifuged*. After resuspension in PBS, cells were counted (median cells alive: 7 x $10^6$ PBMCs) and the volume of each donor was distributed equally to three FACS tubes followed by centrifugation*. The supernatant was discarded and the antibody mix for each of the three flow cytometric panels prepared shortly in advance was added to one FACS tube, respectively, keeping in mind the diverging cell count. Cells were incubated in the dark at room temperature for 20 minutes. After addition of PBS the cells were centrifuged* and the supernatant was poured. Cells were fixed with 150µl 4%PFA in PBS and stored at 4°C until analysis. Analysis at the BD LSRFortessa was begun within 4 hours, with one exception (which also included measurement at a different BD LSRFortessa).
**centrifugation: 7 minutes at 500g at room temperature.*

**Supplemental table 1: Antibodies, fluorochromes, concentrations.** *(*) The stainings for the following antigens were excluded from further analysis, as the measured signal did not show conclusive expression.*

| antigen | fluorochrome | clone | company | lot no. | concentration | dilution | Panel |
|---|---|---|---|---|---|---|---|
| LIVE/DEAD fixable NEAR-IR | | | Thermofisher | 1911161 | | 0,2:100 | Backbone |
| CD3 | AF700 | SK7 | Biolegend | B224024, B238173, B245810 | 200 µg/ml | 3:100 | Backbone |
| CD14 | BUV395 | MφP9 | BD Biosciences | 6259651, 7138976 | 100 µg/ml | 2:100 | Backbone |
| CD16 | BUV737 | 3G8 | BD Biosciences | 7117668 | 400 µg/ml | 1:100 | Backbone |
| CD20 | AF700 | 2H7 | Biolegend | B230880, B243909 | 0.5 mg/ml | 1:100 | Backbone |
| CD56 | AF700 | 5.1H11 | Biolegend | B187698, B234149 | 200 µg/ml | 3:100 | Backbone |
| HLA-DR | BV510 | L243 | Biolegend | B218728, B239989 | 120 µg/ml | 1:100 | Backbone |
| CD11a | APC | HI111 (RUO) | BD Biosciences | 7082913 | 6.25 µg/ml | 7:100 | 1 |
| CD11b | BV785 | M1/70 | Biolegend | B239816 | 100 µg/ml | 1:100 | 1 |
| CD31 | BV605 | WM59 | Biolegend | B229210, B229211 | 50 µg/ml | 5:100 | 1 |
| CD49d | BV711 | 9F10 | Biolegend | B236229 | 50 µg/ml | 1:100 | 1 |
| CD54 | FITC | BBIG-I1 (11C81) | R&D | LAA0816021 | 25 µg/ml | 7:100 | 1 |
| CD192/ CCR2 | BV421 | K036C2 | Biolegend | B226228, B238777 | 80 µg/ml | 1:100 | 1 |
| CD195/ CCR5 | PE | J418F1 | Biolegend | B234453 | 100 µg/ml | 1:100 | 1 |
| CX3CR1 | PE/Cy7 | 2A9-1 | Biolegend | B238466, B218735 | 200 µg/ml | 3,5:100 | 1 |
| CD32 | BV786 | FLI8.26 | BD Biosciences | 7131634 | 50 µg/ml | 2,5:100 | 2 |
| CD64 | BV605 | 10.1 | Biolegend | B203366, B243084 | 100 µg/ml | 2,5:100 | 2 |
| CD115 | PE-Cy7 | 9-4D2-1E4 | Biolegend | B223791 | 400 µg/ml | 1:100 | 2 |
| CD116 | BV421 | hGMCSFR-M1 (RUO) | BD Biosciences | 4108838 | 0.2mg/ml | 2:100 | 2 |
| CD119 | FITC | 92101 | R&D | LAS0317011 | 25 µg/ml | 5:100 | 2 |
| CD132 | AF647 | TUGh4 (RUO | BD Biosciences | 7132687 | 100 µg/ml | 1:100 | 2 |
| *CD274 ** | *BV711* | *10F.9G2* | *Biolegend* | *B232588* | *100 µg/ml* | *2,5:100* | *2* |
| CD284/ TLR4 | PE | TF901 (RUO) | BD Biosciences | 6140828, 7138639 | 50 µg/ml | 2:100 | 2 |
| CD11c | BV711 | B-Ly6 | BD Biosciences | 5267744/ 7299973 | 100 µg/ml / 200 µg/ml | 3:100 / 1,5:100 | 3 |
| CD33 | PE/Cy7 | P67.6 | Biolegend | B225754, B244642 | 200 µg/ml | 2,5:100 | 3 |
| CD39 | BV421 | A1 | Biolegend | B223806, B242450 | 100 µg/ml | 2:100 | 3 |
| *CD69** | *BV786* | *FN50* | *Biolegend* | *B237054* | *80 µg/ml* | *2,5:100* | *3* |
| *CD73** | *APC* | *AD2* | *Biolegend* | *B218129* | *100 µg/ml* | *1:100* | *3* |
| CD163 | BV605 | GHI/61 | Biolegend | B216753, B232232 | 150 µg/ml | 3:100 | 3 |
| CD172a/b | PE | SE5A5 | Biolegend | B221671 | 100 µg/ml | 1:100 | 3 |
| Slan | FITC | DD-1 | Miltenyi | 5170814141 | 22µg/ml | 1:100 | 3 |

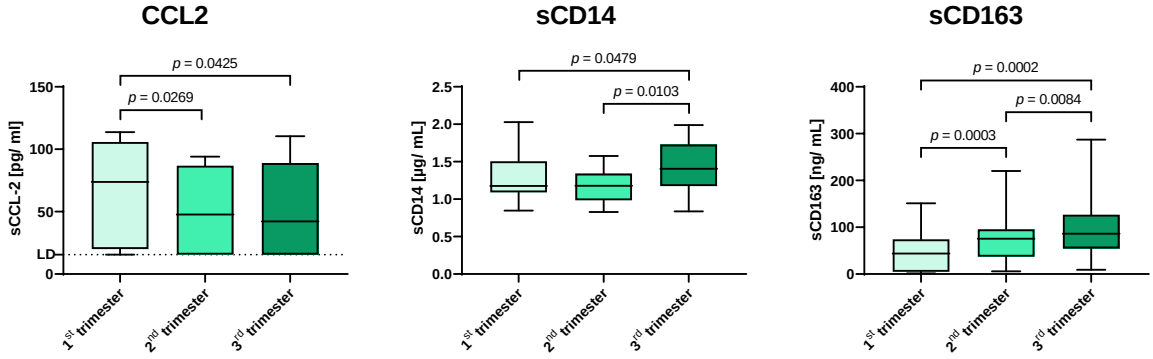
**Supplemental table 2: Function of and references for choosing the analyzed surface markers.** *(*) The stainings for the following antigens were excluded from further analysis, as the measured signal did not show conclusive expression.*

| **antigen** | **function** | **Selection of references** (findings referring to analysis of monocytes) |
|---|---|---|
| CD3 | T cell co-receptor. | |
| CD14 | Glycosylphosphatidylinositol (GPI)-anchored receptor on the surface of myelomonocytic cells, mediates innate immune activation by acting as an independent pattern recognition receptor and as a co-receptor for several Toll-like Receptors by binding microbial products such as LPS. Involved in regulating metabolism, particularly insulin resistance and lipid metabolism. | |
| CD16 | Fcγ receptor III, binds IgG with low affinity and is expressed on natural killer cells, monocytes/ macrophages and neutrophils. Mediates antibody-dependent cellular cytotoxicity. | |
| CD20 | B-lymphocyte surface antigen, expressed in all developmental stages of B cells except from early pro-B cells and plasma cells. | |
| CD56 | Neural cell adhesion molecule (NCAM), in peripheral blood predominantly expressed in natural killer cells. | |
| HLA-DR | MHC class II cell surface receptor, expressed on antigen presenting cells. | |
| CD11a | Adhesive protein subunit (integrin alpha L subunit), association with CD18 forms the integrin lymphocyte function-associated antigen 1 (LFA-1) on leukocytes. | CD11a surface expression was reported to increase throughout pregnancy (1, 2) or to decrease prior to term (3). |
| CD11b | Adhesive protein subunit (integrin alpha M subunit), association with CD18 forms Macrophage-1 antigen (Mac-1), an integrin mainly found in myeloid cells. | CD11b surface expression was reported to increase throughout pregnancy (3-5), or only at labor (1). One study did not find alterations (6). |
| CD31 | Platelet endothelial cell adhesion molecule (PECAM-1), contributes to wound healing, angiogenesis and indispensably to leukocyte diapedesis under most inflammatory conditions. | CD31 surface expression was reported to increase towards the end of pregnancy (3). |
| CD49d | Adhesive protein subunit (integrin α4 subunit), association with integrin β-1 subunit forms VLA-4 or association with β-7 subunit. Both heterodimers are receptors for fibronectin and VCAM-1. Integrin α-4/β-7 binds to mucosal addressin cell adhesion molecule present in lymphatic mucosal tissue and forms a ternary complex with CX3CR1 and CX3CL1. | Analysis of CD49d surface expression throughout pregnancy yielded contradictory results, ranging from increase (7) over non-significant changes (1, 4, 5) to decrease (3). |
| CD54 | Ig-like cell adhesion molecule CD54 or ICAM-1, expressed on leukocytes and endothelial cells, upregulation by proinflammatory stimuli. Among its ligands are LFA-1, Mac-1 and fibrinogen. Involved in leukocyte extravasation to sites of inflammation and as a costimulatory molecule in antigen presentation. | CD54 surface expression was reported to increase throughout pregnancy (1, 3, 7, 8). |
| CD192/ CCR2 | C-C motif chemokine receptor 2 is receptor to CCL2 (or monocyte chemoattractant protein 1), CCL7 and CCL8 act as partial agonists. CCR2 induces monocyte recruitment to sites of inflammation and appears to be required for monocyte release from the bone marrow into the circulation. CCR2 expression is highest on classical and lowest on nonclassical monocytes. | CCR2 surface expression was reported to be decreased (3) or unaltered (9) throughout pregnancy. A correlation of gestational age to CCR2 expression with an increase at term labor was found (6). |
| CD195/ CCR5 | C-C motif chemokine receptor 5, promotes migration, differentiation and proliferation following ligation to its full agonists CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES). CCR5 is expressed on T lymphocytes, monocytes/ macrophages, NK cells and dendritic cells and serves as a co-receptor for HIV-1 cell entry. | $CCR5^+$ monocytes were reported to be decreased in pregnancy, whereas the CCR5 MFI was increased in preeclampsia on intermediate monocytes (9). |
| CX3CR1 | CX3C chemokine receptor, fractalkine receptor or G-protein coupled receptor 13, induces firm adhesion and migration upon interaction with its ligand CX3CL1 (fractalkine, expressed by activated endothelial cells and neural tissue). | CX3CR1 surface expression was reported to increase with maturation from classical over intermediate to nonclassical monocytes (10-12), gene expression was highest in classical (13) or nonclassical monocytes (10). |
| CD32 | Fcγ receptor II, binds IgG with low affinity and is expressed on monocytes/macrophages, B cells, granulocytes and platelets. CD32 can either be activating (CD32a, CD32c) or inhibitory (CD32b), depending on the presence of immunoreceptor tyrosine-based activatory or inhibitory motifs. | CD32 surface expression was reported to increase throughout pregnancy (14). |
| CD64 | Fcγ receptor I, binds IgG with high affinity and is constitutively expressed on monocytes. Ligation can induce phagocytosis, uptake of immune complexes, increased MHC II surface expression and regulates the secretion of inflammatory cytokines and reactive oxygen species. | CD64 surface and mRNA expression were reported to increase throughout pregnancy (1, 4, 5, 14, 15). |
| CD115 | Colony stimulating factor 1 receptor (CSF1R), binds macrophage colony-stimulating factor (M-CSF) and IL-34. M-CSF predominantly regulates myeloid differentiation during homeostasis. CD115 is expressed on monocytes/macrophages, dendritic cells and osteoclasts. It promotes monocyte survival and differentiation into macrophages. | CD115 expression was reported to increase with monocyte maturation with significantly higher gene and surface expression in nonclassical (10, 16) or significantly higher surface expression in $CD14^+CD16^+CCR2^+$ monocytes (17). |

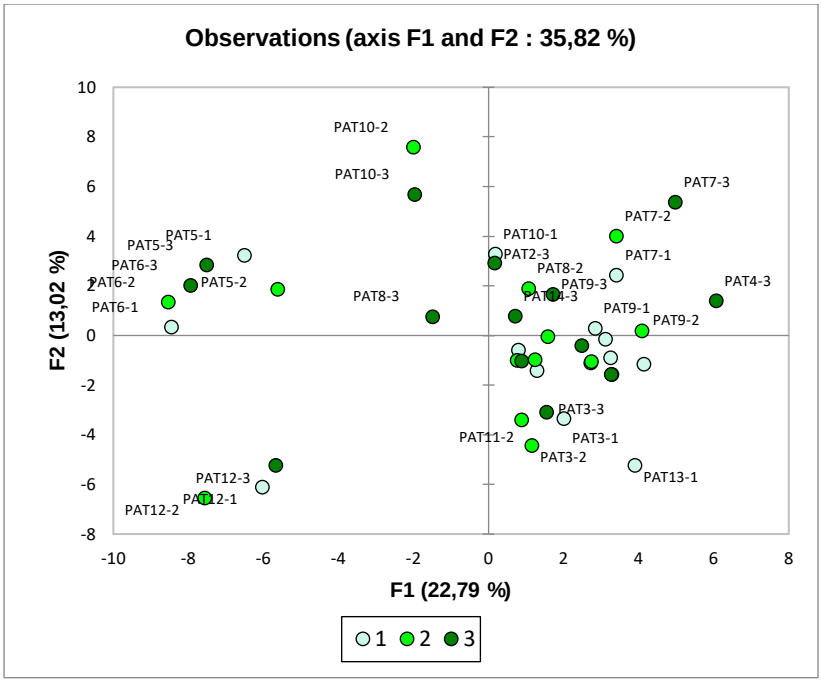
| | | |
|---|---|---|
| CD116 | GM-CSFRα chain, the ligand-specific subunit of the granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor. GM-CSF induces monocyte/ macrophage maturation and effector functions in inflammatory states. | CD116 surface expression was reported to decline with monocyte maturation (18). |
| CD119 | IFN-γ R α chain or IFN-γRI, belongs to the class II cytokine receptor family. The IFN-γ receptor is expressed on most nucleated cells and promotes monocyte activation upon stimulation. | CD119 surface expression was reported to increase in third trimester pregnant women (3). |
| CD132 | Common gamma chain γc, essentially participates in the signaling of the type I cytokine-receptors for IL-2, -4, -7, -9, -15 and -21. Required for assembly of the IL-2 and the IL-4 receptor in monocytes. Signaling via these receptors controls the synthesis of cytokines like IL-1β, IL-8, IL-10, IL-12 and TNF-α; IL-2 induces monocyte activation, IL-4 combined with GM-CSF leads to differentiation of classical monocytes into monocyte-derived dendritic cells. Mutations of the γc gene lead to X-linked severe combined immunodeficiency. | CD132 surface expression was reported to be significantly higher in nonclassical monocytes (19). |
| *CD274 ** | *Programmed death-ligand 1* | |
| CD284/ TLR4 | Pattern recognition receptor recognizing LPS (component of the cell wall of Gram-negative bacteria) and endogenous ligands like extracellular matrix proteins in a complex with myeloid differentiation factor 2. CD14 serves as a coreceptor by inducing TLR4 endocytosis. Signaling results in production of IL-6, TNF-α, IFNβ, IP-10. In blood mainly expressed on monocytes. | TLR4 surface expression was reported to be unaltered (20), increased upon LPS stimulation (21) or decreased (9) throughout pregnancy. |
| CD11c | Adhesive protein subunit (integrin subunit αX), association with CD18 forms complement receptor 4 reported to bind e.g. complement fragment iC3b and to mediate phagocytosis in vitro. | CD11c surface expression was reported to be increased in third trimester pregnant women (2, 3, 22). |
| CD33 | CD33 or sialic acid binding Ig-like lectin 3 (Siglec-3) is an immune regulatory receptor involved in cell-cell interaction and modulation of innate and adaptive effector function by sialic acid recognition, which is present on many human pathogens. CD33 is a marker of myeloid cells and expresses immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which inhibit activation and induce pro-inflammatory cytokine secretion. It furthermore represents an endocytic receptor for sialylated antigens. | Surface and gene expression of CD33 is highest in the classical, lower in the intermediate and lowest in the nonclassical monocyte subset (10, 13, 23, 24). |
| CD39 | The ecto-nucleoside triphosphate diphosphohydrolase 1 CD39 is an integral member of the purinergic system by degrading extracellular ATP into AMP. AMP is then further converted into adenosine by CD73. Thereby immune cells switch from ATP-induced pro-inflammatory activation to an adenosine-driven anti-inflammatory state. | CD39 surface expression in non-pregnant women is upregulated by co-culture with the first trimester trophoblast cell line Swan-71(25). CD39 expression was reported to be downregulated in preeclamptic placentas (26). |
| *CD69** | *C-Type lectin protein, transiently expressed on activated leukocytes and serving as an early activation marker.* | |
| *CD73** | *The ecto-5'-nucleotidase CD73 is an integral member of the purinergic system by degrading extracellular AMP into adenosine. Thereby immune cells switch from ATP-induced pro-inflammatory activation to an adenosine-driven anti-inflammatory state* | |
| CD163 | Scavenger receptor and marker for the monocyte/ macrophage lineage, which binds haptoglobin-hemoglobin complexes and tumour necrosis factor-like weak inducer of apoptosis (TWEAK) and contributes to the resolution of inflammation. | CD163 surface expression was reported to decrease with monocyte maturation (10, 17, 27) and to be decreased in preeclampsia (28). |
| CD172a/b | CD172a or signal regulatory protein (SIRP) α belongs to the immunoregulatory SIRP family and is expressed on myeloid cells and neurons. It contains ITIMs and inhibits cell activity. Ligation with CD47 expressed on nearly all cells or the soluble surfactant proteins A and D reduces monocytic production of TNFα. SIRPβ in contrast mediates cellular activation.<br>The clone used in our experiments reacts with CD172a and has weak cross-reaction with CD172b. | CD172a gene expression was reported to decrease (13), surface expression to increase from classical over intermediate to non-classical monocytes (12). |
| Slan | 6-Sulfo LacNAc, a carbohydrate residue linked to the P-selectin glycoprotein ligand 1, characterizes a distinct subset of nonclassical monocytes. The molecule's exact functions and ligands are still unknown. | Slan gene expression was reported to be restricted to nonclassical monocytes subset and might be used for separation from the intermediate subset (16). |

**Supplemental table 3: Participant characteristics.** Subgroup PRINCE Cohort for functional studies. 21 healthy, pregnant women with natural conception and singleton pregnancies were enrolled. Age is depicted as median [interquartile range – IQR]. Weight and gravity is depicted as percentage value with absolute frequencies.

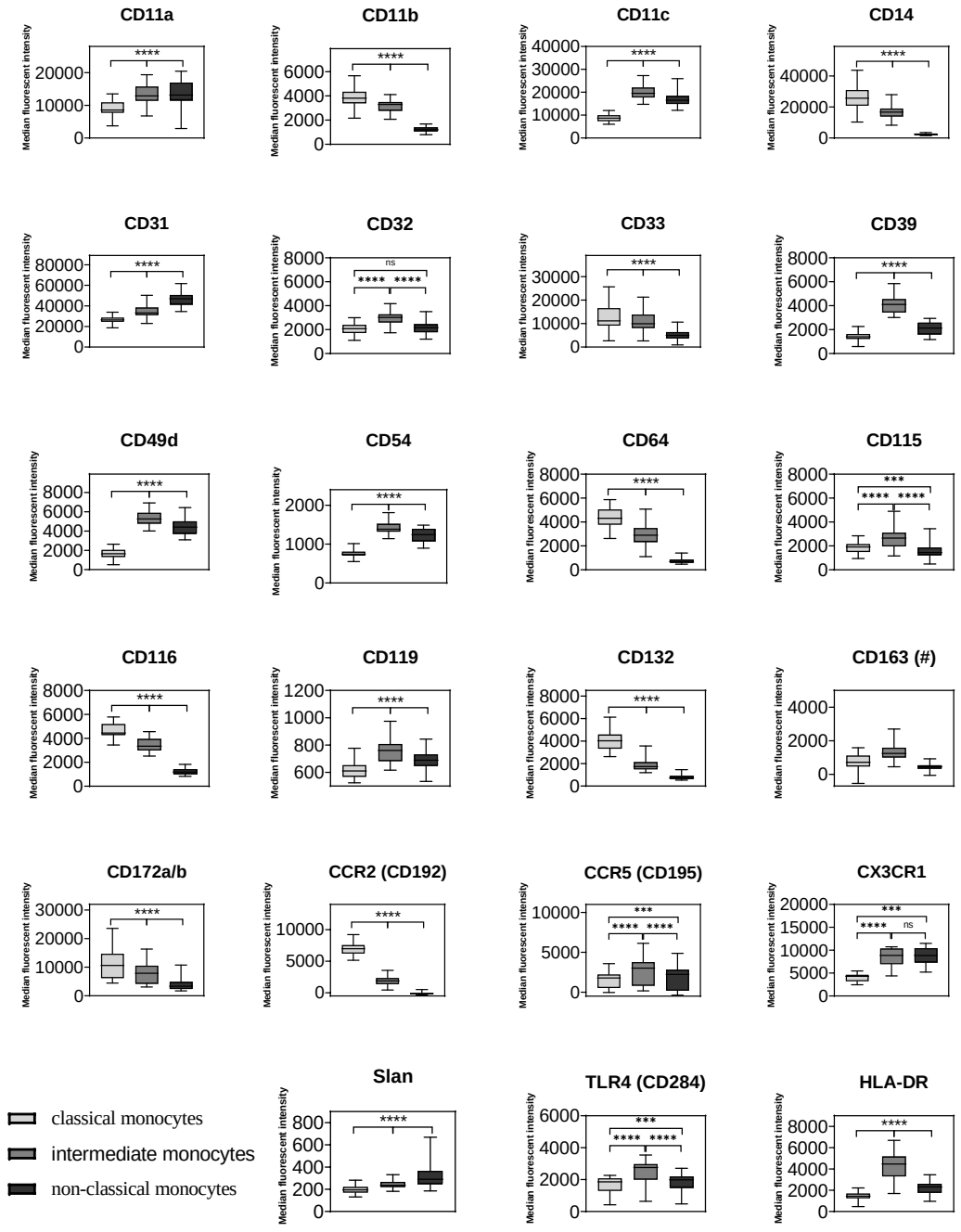
| **PRINCE** | **characteristics** | **total *n* = 21** |
|---|---|---|
| | Age (years) | 32 [28-37] |
| *Weight* | mean BMI ± 1 SD (kg/m2) | 25.1 (±4.3) |
| | BMI = 18,5 - <25 (kg/m2) | 62.9 % (n = 22) |
| | BMI = 25 - <30 (kg/m2) | 25.7 % (n = 9) |
| | BMI ≥ 30 (kg/m2) | 11.4 % (n = 4) |
| *Gravidity* | Primigravida | 42.9 % (n = 15) |
| | Multigravida | 57.1 % (n = 20) |



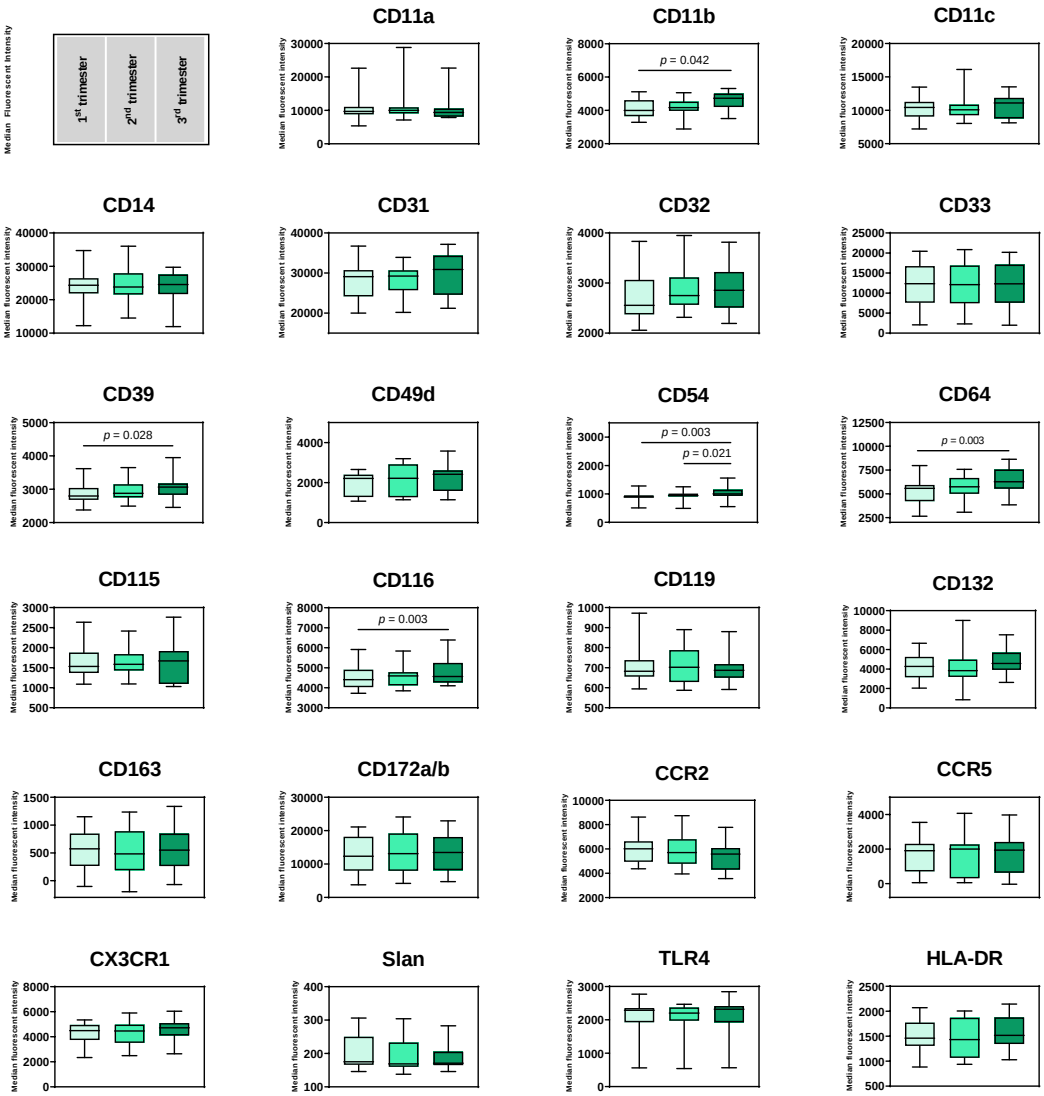

**Supplemental figure 1: CCL2, sCD14 and sCD163 levels in sera from pregnant women during the course of pregnancy.** ELISAs were performed in accordance to the manufacturer's protocol. (LD = limit of detection; Wilcoxon matched-pairs signed rank test, FDR-adjusted p-values)




**Supplemental figure 2: PCA of pregnant women based on monocyte phenotypes.** Cryopreserved PBMCs from 15 pregnant women from each trimester of pregnancy were surface stained. Monocytes were grouped according to their CD14 and CD16 expression into classical, intermediate and non-classical monocytes (figure 1A). The median fluorescence intensity was determined for 23 surface molecules expressed on monocytes and used for principal component analysis. The projection of the participants according to the first two principal components are shown. The indicators 1, 2, 3 (with different shades of green) correspond to sampling in the first, second and third pregnancy trimester, respectively.




**Supplemental figure 3: Marker distribution on monocyte subsets.** Cryopreserved PBMCs were surface stained (n=30, 15 non-pregnant females, 15 males). Monocytes were grouped according to their CD14 and CD16 expression into classical, intermediate and non-classical monocytes (figure 1A). The median fluorescence intensity is depicted for the respective monocyte subsets (Wilcoxon signed rank test, FDR-adjusted p-values). #: Differential stability of CD163 on monocyte subsets following cryopreservation did not allow for comparison among monocyte subsets.




**Supplemental figure 4: Median fluorescence intensity of analyzed surface antigens on bulk CD14$^{pos}$ monocytes.** Cryopreserved PBMCs from 15 pregnant women from each trimester of pregnancy were surface stained. Monocytes were defined as lin$^{neg}$HLA-DR$^{pos}$CD14$^{pos}$. The median fluorescence intensity is depicted for the three trimesters of pregnancy (Wilcoxon signed rank test, FDR-adjusted p-values).

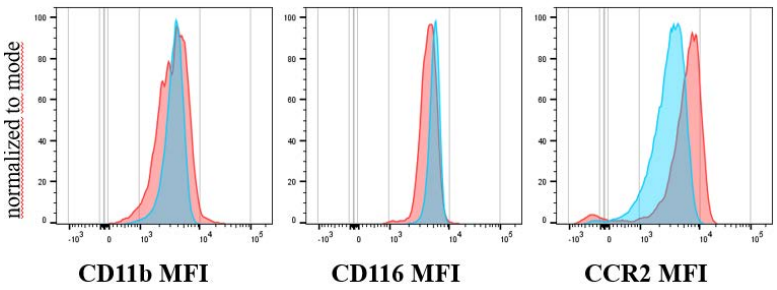
**Supplemental table 4: Median fluorescence intensities (MFI) of analyzed antigens on the respective monocyte subset throughout the course of pregnancy.**
PBMCs from pregnant women were stained and analyzed using multiparameter flow cytometry. Statistical analyses of the data were conducted using GraphPad Prism 7.04. Depicted is the median of the MFIs of 15 pregnant females. Wilcoxon matched-pairs signed rank tests were applied for paired comparison of the pregnant women. FDR-adjusted p-values. P-values considered significant ($p<0.05$) are highlighted in red.

| | | Bulk monocytes | | | Classical monocytes | | | Intermediate monocytes | | | Non-classical monocytes | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Antigen** | **Tri** | **MFI** | **comparison** | ***p-values*** | **MFI** | **comparison** | ***p-values*** | **MFI** | **comparison** | ***p-values*** | **MFI** | **comparison** | ***p-values*** |
| **CD11a** | **T1** | 9685 | T1/T2 | 0,7767 | 9301 | T1/T2 | 0,7913 | 15171 | T1/T2 | 0,8520 | 16613 | T1/T2 | 0,5842 |
| | **T2** | 10019 | T2/T3 | 0,7435 | 9664 | T2/T3 | 0,6019 | 14550 | T2/T3 | 0,2715 | 16382 | T2/T3 | 0,2208 |
| | **T3** | 9385 | T1/T3 | 0,8520 | 9034 | T1/T3 | 0,5515 | 13989 | T1/T3 | 0,3355 | 14822 | T1/T3 | 0,3804 |
| **CD11b** | **T1** | 3999 | T1/T2 | 0,8520 | 4049 | T1/T2 | 0,8704 | 3761 | T1/T2 | 0,6489 | 1258 | T1/T2 | 0,6489 |
| | **T2** | 4167 | T2/T3 | 0,1458 | 4263 | T2/T3 | 0,1458 | 4082 | T2/T3 | **0,0417** | 1218 | T2/T3 | 0,7913 |
| | **T3** | 4731 | T1/T3 | **0,0417** | 4903 | T1/T3 | **0,0315** | 4380 | T1/T3 | **0,0029** | 1223 | T1/T3 | 0,1730 |
| **CD11c** | **T1** | 10435 | T1/T2 | 0,9720 | 9817 | T1/T2 | 1,0000 | 20961 | T1/T2 | 0,8630 | 19668 | T1/T2 | 0,8520 |
| | **T2** | 10087 | T2/T3 | 0,6289 | 9406 | T2/T3 | 0,8055 | 21923 | T2/T3 | 0,5122 | 20232 | T2/T3 | 0,9720 |
| | **T3** | 11095 | T1/T3 | 0,3636 | 10064 | T1/T3 | 0,8704 | 19854 | T1/T3 | 0,1568 | 20520 | T1/T3 | 0,7913 |
| **CD31** | **T1** | 29097 | T1/T2 | 0,8899 | 28751 | T1/T2 | 0,8630 | 31869 | T1/T2 | 0,5710 | 43378 | T1/T2 | 0,8520 |
| | **T2** | 29236 | T2/T3 | 0,6796 | 28820 | T2/T3 | 0,6489 | 29659 | T2/T3 | 0,9720 | 39957 | T2/T3 | 0,9721 |
| | **T3** | 30891 | T1/T3 | 0,2816 | 30891 | T1/T3 | 0,1730 | 31114 | T1/T3 | 0,6019 | 41531 | T1/T3 | 0,2015 |
| **CD32** | **T1** | 2554 | T1/T2 | 0,1856 | 2524 | T1/T2 | 0,1730 | 3427 | T1/T2 | 0,8055 | 2107 | T1/T2 | 0,8704 |
| | **T2** | 2750 | T2/T3 | 0,8055 | 2744 | T2/T3 | 0,8704 | 3568 | T2/T3 | 0,9833 | 1998 | T2/T3 | 0,4448 |
| | **T3** | 2854 | T1/T3 | 0,2471 | 2810 | T1/T3 | 0,2816 | 3716 | T1/T3 | 0,6289 | 1828 | T1/T3 | 0,1231 |
| **CD33** | **T1** | 12325 | T1/T2 | 0,4096 | 12524 | T1/T2 | 0,6019 | 11351 | T1/T2 | 0,6475 | 4913 | T1/T2 | 0,2208 |
| | **T2** | 12101 | T2/T3 | 0,9720 | 12381 | T2/T3 | 1,0000 | 12101 | T2/T3 | 0,2816 | 4791 | T2/T3 | 0,8311 |
| | **T3** | 12296 | T1/T3 | 0,7140 | 12467 | T1/T3 | 0,7913 | 12268 | T1/T3 | 0,0691 | 4934 | T1/T3 | 0,2015 |
| **CD39** | **T1** | 2799 | T1/T2 | 0,6259 | 1360 | T1/T2 | 0,9126 | 4185 | T1/T2 | 0,7767 | 2266 | T1/T2 | 0,9609 |
| | **T2** | 2877 | T2/T3 | 0,2015 | 1337 | T2/T3 | 0,8348 | 4290 | T2/T3 | 0,7428 | 2160 | T2/T3 | 0,9126 |
| | **T3** | 3064 | T1/T3 | **0,0280** | 1397 | T1/T3 | 0,7913 | 4281 | T1/T3 | 0,9833 | 2471 | T1/T3 | 0,8661 |
| **CD49d** | **T1** | 2210 | T1/T2 | 0,3804 | 1987 | T1/T2 | 0,3355 | 5762 | T1/T2 | 0,9609 | 5313 | T1/T2 | 0,7140 |
| | **T2** | 2214 | T2/T3 | 0,7767 | 2033 | T2/T3 | 1,0000 | 5799 | T2/T3 | 0,6821 | 5652 | T2/T3 | 0,6019 |
| | **T3** | 2414 | T1/T3 | 0,0811 | 1991 | T1/T3 | 0,2513 | 5474 | T1/T3 | 0,5556 | 5029 | T1/T3 | 0,8055 |
| **CD54** | **T1** | 893 | T1/T2 | 0,4733 | 857 | T1/T2 | 0,5556 | 1561 | T1/T2 | 0,6796 | 1270 | T1/T2 | 0,6350 |
| | **T2** | 943 | T2/T3 | **0,0211** | 897 | T2/T3 | **0,0235** | 1705 | T2/T3 | 0,8630 | 1389 | T2/T3 | 0,8899 |
| | **T3** | 1002 | T1/T3 | **0,0029** | 954 | T1/T3 | **0,0029** | 1702 | T1/T3 | 0,3355 | 1312 | T1/T3 | 0,7140 |
| **CD64** | **T1** | 5580 | T1/T2 | 0,1458 | 5750 | T1/T2 | 0,1777 | 4150 | T1/T2 | 0,0691 | 821 | T1/T2 | 0,4764 |
| | **T2** | 5737 | T2/T3 | 0,0691 | 5862 | T2/T3 | 0,1167 | 5533 | T2/T3 | **0,0151** | 803 | T2/T3 | 0,5777 |
| | **T3** | 6267 | T1/T3 | **0,0029** | 6240 | T1/T3 | **0,0029** | 6896 | T1/T3 | **0,0029** | 853 | T1/T3 | 0,0599 |
| **CD115** | **T1** | 1532 | T1/T2 | 0,9720 | 1495 | T1/T2 | 0,9720 | 2266 | T1/T2 | 1,0000 | 1489 | T1/T2 | 0,3804 |
| | **T2** | 1585 | T2/T3 | 0,9720 | 1535 | T2/T3 | 0,9720 | 2095 | T2/T3 | 0,7785 | 1143 | T2/T3 | 0,6193 |
| | **T3** | 1670 | T1/T3 | 0,9833 | 1576 | T1/T3 | 0,8899 | 2428 | T1/T3 | 0,8704 | 1106 | T1/T3 | 0,3110 |
| **CD116** | **T1** | 4407 | T1/T2 | 0,2816 | 4518 | T1/T2 | 0,3355 | 3504 | T1/T2 | 0,2715 | 1181 | T1/T2 | 0,9338 |
| | **T2** | 4594 | T2/T3 | 0,1856 | 4672 | T2/T3 | 0,1306 | 3910 | T2/T3 | **0,0151** | 1074 | T2/T3 | 0,7140 |
| | **T3** | 4566 | T1/T3 | **0,0029** | 4672 | T1/T3 | **0,0029** | 4116 | T1/T3 | **0,0029** | 1147 | T1/T3 | 1,0000 |
| **CD119** | **T1** | 682 | T1/T2 | 1,0000 | 677 | T1/T2 | 1,0000 | 786 | T1/T2 | 0,9986 | 669 | T1/T2 | 0,8899 |
| | **T2** | 702 | T2/T3 | 0,8899 | 684 | T2/T3 | 0,7913 | 817 | T2/T3 | 0,8899 | 673 | T2/T3 | 0,1545 |
| | **T3** | 687 | T1/T3 | 0,8630 | 676 | T1/T3 | 0,6796 | 803 | T1/T3 | 0,9833 | 652 | T1/T3 | 0,3567 |
| **CD132** | **T1** | 4263 | T1/T2 | 0,8899 | 4389 | T1/T2 | 0,8704 | 2099 | T1/T2 | 0,3355 | 725 | T1/T2 | 0,9833 |
| | **T2** | 3831 | T2/T3 | 0,5122 | 3959 | T2/T3 | 0,4448 | 2539 | T2/T3 | 0,1167 | 730 | T2/T3 | 0,6796 |
| | **T3** | 4575 | T1/T3 | 0,1856 | 4831 | T1/T3 | 0,1568 | 3547 | T1/T3 | **0,0029** | 730 | T1/T3 | 0,9126 |
| **CD163** | **T1** | 574 | T1/T2 | 0,8630 | 552 | T1/T2 | 0,7190 | 1057 | T1/T2 | 0,9126 | 405 | T1/T2 | 0,1568 |
| | **T2** | 483 | T2/T3 | 0,8311 | 445 | T2/T3 | 0,9720 | 920 | T2/T3 | 0,6796 | 314 | T2/T3 | 0,7963 |
| | **T3** | 550 | T1/T3 | 0,8661 | 538 | T1/T3 | 0,9126 | 1145 | T1/T3 | 0,2208 | 321 | T1/T3 | 0,3670 |
| **CD172a/b** | **T1** | 12325 | T1/T2 | 0,1856 | 12786 | T1/T2 | 0,1730 | 10626 | T1/T2 | 0,0993 | 3663 | T1/T2 | 0,6193 |
| | **T2** | 13114 | T2/T3 | 0,8311 | 13544 | T2/T3 | 0,8311 | 12669 | T2/T3 | 0,6796 | 4099 | T2/T3 | 1,0000 |
| | **T3** | 13481 | T1/T3 | 0,2471 | 14021 | T1/T3 | 0,2513 | 13114 | T1/T3 | **0,0061** | 4008 | T1/T3 | 0,8899 |
| **CCR2** | **T1** | 6015 | T1/T2 | 0,8899 | 6213 | T1/T2 | 0,8630 | 2599 | T1/T2 | **0,0417** | -244 | T1/T2 | 0,8520 |
| | **T2** | 5701 | T2/T3 | 0,2816 | 5989 | T2/T3 | 0,3355 | 3716 | T2/T3 | **0,0348** | -220 | T2/T3 | 0,2471 |
| | **T3** | 5580 | T1/T3 | 0,3110 | 5824 | T1/T3 | 0,2816 | 4254 | T1/T3 | **0,0029** | -262 | T1/T3 | 0,8630 |
| **CCR5** | **T1** | 1906 | T1/T2 | 0,9833 | 1874 | T1/T2 | 0,9986 | 2761 | T1/T2 | 0,7493 | 2111 | T1/T2 | 0,2816 |
| | **T2** | 2002 | T2/T3 | 0,6796 | 1935 | T2/T3 | 0,7140 | 2554 | T2/T3 | 0,8704 | 2029 | T2/T3 | 1,0000 |
| | **T3** | 1939 | T1/T3 | 0,7767 | 1877 | T1/T3 | 0,8899 | 2882 | T1/T3 | 0,8704 | 1839 | T1/T3 | 0,3804 |
| **CX3CR1** | **T1** | 4490 | T1/T2 | 0,9986 | 4142 | T1/T2 | 0,7913 | 8486 | T1/T2 | 0,9833 | 8873 | T1/T2 | 0,9720 |
| | **T2** | 4462 | T2/T3 | 0,2015 | 4159 | T2/T3 | 0,2816 | 8933 | T2/T3 | 0,9833 | 8993 | T2/T3 | 0,9720 |
| | **T3** | 4721 | T1/T3 | 0,3804 | 4290 | T1/T3 | 0,5515 | 8658 | T1/T3 | 0,8055 | 9449 | T1/T3 | 0,9126 |
| **Slan** | **T1** | 175 | T1/T2 | 0,9720 | 173 | T1/T2 | 0,9152 | 209 | T1/T2 | 1,0000 | 314 | T1/T2 | 0,8520 |
| | **T2** | 169 | T2/T3 | 0,8899 | 164 | T2/T3 | 0,7991 | 209 | T2/T3 | 0,8570 | 352 | T2/T3 | 0,6489 |
| | **T3** | 171 | T1/T3 | 0,9126 | 165 | T1/T3 | 0,5777 | 212 | T1/T3 | 0,9974 | 328 | T1/T3 | 0,8055 |
| **TLR4** | **T1** | 2288 | T1/T2 | 0,3670 | 2236 | T1/T2 | 0,2816 | 3359 | T1/T2 | 0,6193 | 2319 | T1/T2 | 1,0000 |
| | **T2** | 2202 | T2/T3 | 0,3433 | 2152 | T2/T3 | 0,6289 | 3379 | T2/T3 | 0,6489 | 2305 | T2/T3 | 0,9338 |
| | **T3** | 2319 | T1/T3 | 0,8153 | 2249 | T1/T3 | 1,0000 | 3406 | T1/T3 | 0,5515 | 2279 | T1/T3 | 0,8311 |

**Supplemental table 5: Surface marker expression vs. hormone correlation.**
Correlations were calculated using GraphPad Prism 8.0.1. Analysis performed was Spearman correlation. FDR-adjusted p-values. P-values considered significant ($p<0.05$) are highlighted in red.

| | | hormone | | CD11b | CD14 | CD54 | CD64 | CD116 | CD132 | CD172a/b | CCR2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| classical monocytes | 1 Trimester | 17-β Estradiol | r | 0.1429 | -0.05501 | -0.2009 | 0.1604 | 0.2332 | -0.367 | 0.1077 | -0.07692 |
| | | | p | 0,8899 | 0,9720 | 0,8311 | 0,8704 | 0,7913 | 0,5595 | 0,9338 | 0,9720 |
| | | Progesterone | r | -0.2352 | 0,0242 | -0.0287 | -0.1385 | -0.4158 | 0.06813 | 0.002198 | 0.156 |
| | | | p | 0,7913 | 0,9989 | 0,9986 | 0,8899 | 0,4548 | 0,9720 | 1,0000 | 0,8704 |
| | | hCG | r | -0.4769 | 0.1012 | -0.02428 | -0.1516 | -0.7305 | 0.1736 | 0.2659 | 0.06813 |
| | | | p | 0,3433 | 0,9403 | 0,9991 | 0,8741 | **0,0480** | 0,8630 | 0,7428 | 0,9720 |
| | 2 Trimester | 17-β Estradiol | r | 0.3179 | -0.3179 | -0.02857 | -0.09286 | 0.2214 | -0.4393 | 0.1679 | 0.5536 |
| | | | p | 0,6259 | 0,6259 | 0,9986 | 0,9483 | 0,7963 | 0,3804 | 0,8630 | 0,2015 |
| | | Progesterone | r | -0.625 | -0.1893 | -0.1929 | -0.02143 | -0.3607 | 0.1607 | 0.1071 | 0.3607 |
| | | | p | 0,1306 | 0,8348 | 0,8311 | 1,0000 | 0,5531 | 0,8660 | 0,9274 | 0,5531 |
| | | hCG | r | -0.775 | -0.06429 | -0.1143 | 0.2571 | -0.675 | 0.2607 | 0.2179 | -0.08571 |
| | | | p | **0,0163** | 0,9720 | 0,9126 | 0,7408 | 0,0980 | 0,7339 | 0,7991 | 0,9609 |
| | 3 Trimester | 17-β Estradiol | r | -0.0983 | -0.2502 | 0.1536 | -0.05714 | 0.1821 | -0.01429 | -0.1929 | -0.07857 |
| | | | p | 0,9387 | 0,7493 | 0,8704 | 0,9720 | 0,8520 | 1,0000 | 0,8311 | 0,9706 |
| | | Progesterone | r | -0.4254 | -0.2466 | 0.2107 | -0.08571 | -0.075 | -0.05357 | -0.06786 | -0.03929 |
| | | | p | 0,3954 | 0,7563 | 0,8055 | 0,9609 | 0,9720 | 0,9720 | 0,9720 | 0,9833 |
| | | hCG | r | -0.445 | -0.07507 | -0.3143 | 0.3464 | -0.45 | 0.2321 | -0.09643 | 0.1786 |
| | | | p | 0,3670 | 0,9720 | 0,6302 | 0,5743 | 0,3636 | 0,7866 | 0,9403 | 0,8520 |
| intermediate monocytes | 1 Trimester | 17-β Estradiol | r | -0.01101 | -0.03297 | -0.2791 | 0.02418 | 0.1736 | -0.2835 | 0.05501 | -0.02857 |
| | | | p | 1,0000 | 0,9974 | 0,7177 | 0,9991 | 0,8630 | 0,7140 | 0,9720 | 0,9986 |
| | | Progesterone | r | -0.1123 | 0.06374 | 0.1297 | 0.08571 | -0.1736 | 0.1648 | 0.0484 | 0.2527 |
| | | | p | 0,9263 | 0,9720 | 0,9067 | 0,9691 | 0,8630 | 0,8661 | 0,9811 | 0,7713 |
| | | hCG | r | -0.304 | -0.01099 | 0.06813 | 0.03297 | -0.4989 | 0.1824 | 0.3058 | 0.1209 |
| | | | p | 0,6717 | 1,0000 | 0,9720 | 0,9974 | 0,3110 | 0,8570 | 0,6650 | 0,9126 |
| | 2 Trimester | 17-β Estradiol | r | -0.3628 | -0.3324 | -0.3107 | -0.3038 | -0.1107 | -0.5571 | 0.06071 | 0.01429 |
| | | | p | 0,5496 | 0,6000 | 0,6350 | 0,6478 | 0,9207 | 0,2015 | 0,9720 | 1,0000 |
| | | Progesterone | r | -0.311 | -0.1037 | -0.01071 | -0.01609 | -0.2821 | 0.1714 | 0.1179 | 0.35 |
| | | | p | 0,6349 | 0,9324 | 1,0000 | 1,0000 | 0,6821 | 0,8630 | 0,9126 | 0,5631 |
| | | hCG | r | -0.05004 | 0.2145 | -0.05357 | 0.3253 | -0.2893 | 0.3786 | 0.3 | 0.3357 |
| | | | p | 0,9743 | 0,8055 | 0,9720 | 0,6141 | 0,6796 | 0,5122 | 0,6489 | 0,5934 |
| | 3 Trimester | 17-β Estradiol | r | -0.01429 | 0.05714 | 0.3 | 0.07864 | 0.2821 | 0.003571 | -0.09651 | 0.1893 |
| | | | p | 1,0000 | 0,9720 | 0,6489 | 0,9706 | 0,6821 | 1,0000 | 0,9403 | 0,8348 |
| | | Progesterone | r | -0.3536 | -0.08929 | 0.3464 | 0.02502 | 0.05714 | 0.02857 | -0.04111 | 0.225 |
| | | | p | 0,5595 | 0,9584 | 0,5687 | 0,9986 | 0,9720 | 0,9986 | 0,9833 | 0,7913 |
| | | hCG | r | -0.1679 | 0.2321 | -0.1464 | 0.5183 | 0.1714 | 0.4071 | 0.008937 | 0.7714 |
| | | | p | 0,8630 | 0,7866 | 0,8719 | 0,2513 | 0,8630 | 0,4443 | 1,0000 | **0,0273** |
| non-classical monocytes | 1 Trimester | 17-β Estradiol | r | 0.156 | 0.0989 | -0.363 | 0.06374 | 0.06167 | -0.00659 | 0.1297 | 0.03297 |
| | | | p | 0,8704 | 0,9440 | 0,5631 | 0,9720 | 0,9720 | 1,0000 | 0,9067 | 0,9974 |
| | | Progesterone | r | -0.4769 | -0.3363 | -0.08141 | -0.4462 | -0.2445 | -0.222 | -0.222 | -0.3934 |
| | | | p | 0,3433 | 0,6193 | 0,9706 | 0,3850 | 0,7785 | 0,8055 | 0,8055 | 0,5122 |
| | | hCG | r | -0.5165 | -0.433 | -0.1298 | -0.2703 | -0.6498 | 0.1604 | 0.06374 | -0.4242 |
| | | | p | 0,2816 | 0,4190 | 0,9055 | 0,7376 | 0,1267 | 0,8704 | 0,9720 | 0,4435 |
| | 2 Trimester | 17-β Estradiol | r | 0.2071 | -0.00714 | -0.3571 | -0.2179 | 0.1286 | -0.5429 | 0.05357 | 0.1071 |
| | | | p | 0,8084 | 1,0000 | 0,5553 | 0,7991 | 0,8979 | 0,2208 | 0,9720 | 0,9274 |
| | | Progesterone | r | -0.5571 | 0.2036 | -0.1143 | -0.1286 | -0.2036 | 0.15 | -0.2107 | 0.06071 |
| | | | p | 0,2015 | 0,8155 | 0,9126 | 0,8979 | 0,8155 | 0,8704 | 0,8055 | 0,9720 |
| | | hCG | r | -0.5893 | 0.1786 | 0.1964 | 0.1179 | -0.4786 | 0.4464 | -0.1643 | -0.2179 |
| | | | p | 0,1629 | 0,8520 | 0,8311 | 0,9126 | 0,3130 | 0,3670 | 0,8630 | 0,7991 |
| | 3 Trimester | 17-β Estradiol | r | 0.1912 | -0.1607 | 0.3607 | 0.09643 | 0.2536 | -0.07328 | -0.1393 | 0.2627 |
| | | | p | 0,8311 | 0,8660 | 0,5515 | 0,9403 | 0,7442 | 0,9720 | 0,8899 | 0,7249 |
| | | Progesterone | r | -0.4897 | -0.4321 | -0.2357 | -0.4964 | -0.05714 | -0.2878 | -0.4857 | -0.3914 |
| | | | p | 0,2896 | 0,3838 | 0,7785 | 0,2816 | 0,9720 | 0,6796 | 0,3005 | 0,4762 |
| | | hCG | r | -0.5022 | -0.01071 | -0.375 | -0.1143 | -0.03214 | 0.1001 | -0.3214 | -0.5273 |
| | | | p | 0,2795 | 1,0000 | 0,5122 | 0,9126 | 0,9974 | 0,9338 | 0,6212 | 0,2437 |

**Supplemental figure 5: Median fluorescence intensities (MFI) on fresh versus cryopreserved bulk CD14$^{pos}$ monocytes.** Fresh and cryopreserved PBMCs from the same healthy non-pregnant individual were surface stained and analyzed following the protocols and gating strategy described in the manuscript. The histograms compare the MFI of chosen surface markers on fresh (red) versus thawed (blue) bulk monocytes.



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## 2. Darstellung der Publikation

### 2.1. Einleitung

Eine Schwangerschaft stellt das maternale Immunsystem vor eine besondere Herausforderung. Zum Zweck ihrer Aufrechterhaltung muss eine Balance zwischen effektiven antimikrobiellen Verteidigungsmechanismen und immunologischer Toleranz gegenüber dem semi-allogenen Fetus herrschen. Eine gesteigerte Responsivität des angeborenen Immunsystems trägt während humaner Schwangerschaften nachweislich zu dieser Balance bei (Sacks et al., 1999, Luppi et al., 2002b). Die physiologische Aktivierung des angeborenen Immunsystems wird im Rahmen der Schwangerschaft durch eine Kombination verschiedener Faktoren induziert, zu welchen die mütterlichen Gestationshormone, der Kontakt der Leukozyten zur Plazenta sowie ihre Interaktion mit löslichen plazentalen Produkten und fetalen Zellen in der Blutstrombahn gehören (Sacks et al., 1999, Veenstra van Nieuwenhoven et al., 2003a).

Monozyten als Teil des angeborenen Immunsystems stellen professionelle Phagozyten im Blutkreislauf dar. Ihre Einteilung in drei Subpopulationen erfolgt anhand der Expression von zwei Cluster of Differentiation (CD) - Molekülen an der Zelloberfläche, dem Pattern Recognition Receptor CD14 und dem FcγIII-Rezeptor CD16. Dadurch lassen sich immunphänotypisch die klassische $CD14^{++}CD16^{-}$, die intermediäre $CD14^{++}CD16^{+}$ und die nicht-klassische $CD14^{+}CD16^{++}$ Monozyten-Subpopulation voneinander abgrenzen (Ziegler-Heitbrock et al., 2010). Diese Subpopulationen scheinen sequenzielle Entwicklungsstufen der Monozytendifferenzierung zu verkörpern (Wong et al., 2011, Patel et al., 2017) und unterscheiden sich in ihrer funktionellen Spezialisierung. Klassische Monozyten sind potente Phagozyten, welche präferenziell auf Stimuli wie Pathogene oder Inflammation reagieren und in Chemotaxis und Gewebereparatur involviert sind; intermediäre Monozyten tragen insbesondere zur Antigenpräsentation, zur Regulation der Apoptose und zur Zytokinsekretion bei, während nicht-klassische Monozyten hochdifferenziert sind, entlang des Endothels patrouillieren und Gene für die Fc-Rezeptor-vermittelte Phagozytose exprimieren (Wong et al., 2011, Gren et al., 2015, Hofer et al., 2015).

Die Aktivierung von Monozyten während der Schwangerschaft induziert beträchtliche Veränderungen der Zusammensetzung der Subpopulationen, des Phänotyps und der Funktion (Sacks et al., 1998, Naccasha et al., 2001, Luppi et al., 2002a, Sacks et al., 2003, Veenstra van Nieuwenhoven et al., 2003b, Holthe et al., 2004, Al-ofi et al., 2012, Melgert et al., 2012, Mikhaylova et al., 2013, Groen et al., 2015, Lampe et al., 2015, Zhang et al., 2017, Ziegler et al., 2018). Ihre Aktivierung kann durch die Phagozytose vom Trophoblasten und vom Fetus stammender Partikel induziert werden (Messerli et al., 2010, Atay et al., 2011, Southcombe et al., 2011, Bianchi et al., 1996). Auch die uteroplazentale Passage scheint Monozyten während der Schwangerschaft zu aktivieren (Mellembakken et al.,

2002). Hohe Konzentrationen von 17β-Östradiol übten in Experimenten anti-inflammatorische Effekte auf Monozyten aus, wobei der Einfluss der Sexualhormone auf die Monozytenaktivierung *in vitro* insgesamt widersprüchlich erscheint (Bouman et al., 2005). Das Ausmaß der Monozytenaktivierung kann mittels löslicher Marker im Blut ermittelt werden. Dazu eignen sich sCD14 und sCD163, welche in der Folge proinflammatorischer Stimuli von der Oberflächenmembran abgestoßen werden (sog. „Shedding“) (Chenevier-Gobeaux et al., 2015, Zhi et al., 2018), sowie CC-Chemokin-Ligand 2 (CCL2), ein Chemokin, welches hauptsächlich von Monozyten und Makrophagen sezerniert wird (Deshmane et al., 2009). Studien zu Monozyten während gesunder Schwangerschaften beschreiben einstimmig deren Aktivierung, jedoch divergieren die Resultate hinsichtlich der begleitenden phänotypischen Veränderungen und der Zytokinproduktion (Faas und de Vos, 2017). Mögliche Ursachen könnten der Zeitpunkt der Probengewinnung, ein Querschnitt-Studiendesign oder methodische Aspekte darstellen. Eine darüber hinaus gesteigerte, dysregulierte Monozytenaktivierung wurde bei Präeklampsie nachgewiesen (Naccasha et al., 2001, Mellembakken et al., 2002, Al-ofi et al., 2012, Faas und de Vos, 2017), einer Schwangerschaftskomplikation, deren Entstehung auf eine gestörte Trophoblastenimplantation zurückgeführt wird. Da Monozyten zu einer erfolgreichen Trophoblastenimplantation und zum Umbau der Spiralarterien (Remodelling) beitragen, an der frühen Pathogenabwehr beteiligt sind und in entzündetes Gewebe migrieren (Veenstra van Nieuwenhoven et al., 2003a, Faas et al., 2014), repräsentieren sie ein wichtiges Forschungsgebiet in der Schwangerschaft.

Im Verlauf der Schwangerschaft werden unterschiedliche immunologische Phasen mit speziell adaptierten Zytokinmilieus durchlaufen, welche die jeweiligen Prozesse der Implantation, der Plazentation, des Wachstums und der Entbindung ermöglichen (Mor und Cardenas, 2010). Im Gegensatz zu immunologischen Studien über Schwangerschaften mit widrigem Ausgang ist die Anzahl longitudinaler Studien zur Biologie gesunder Schwangerschaften während aller immunologischen Phasen limitiert. Insbesondere Studien, die sich auf die Heterogenität der Monozyten-Subpopulationen fokussieren, fehlen, obwohl sich die phänotypischen und funktionellen Charakteristika der Gesamt-Monozyten signifikant verändern (Sacks et al., 1998, Naccasha et al., 2001, Luppi et al., 2002a, Sacks et al., 2003, Veenstra van Nieuwenhoven et al., 2003b, Holthe et al., 2004, Al-ofi et al., 2012, Mikhaylova et al., 2013, Lampe et al., 2015, Zhang et al., 2017, Ziegler et al., 2018). Aus diesem Grund erfolgte eine prospektive Entnahme von Blutproben gesunder Schwangerer während definierter Zeitspannen in jedem Trimenon. Die peripheren Sexualhormone, das monozytäre Aktivitätslevel, die detaillierten phänotypischen Charakteristika der Monozyten-Subpopulationen und das sekretorische Potenzial der Monozyten wurden analysiert. Die Oberflächen-Antigene für die

phänotypische Analyse wurden auf der Basis divergierender Expression entweder auf den Monozyten-Subpopulationen oder in verschiedenen Schwangerschaftsphasen auf den Gesamtmonozyten ausgewählt, um funktionell wichtige Antigene zu untersuchen und um vorherige Ergebnisse auf die Monozyten-Subpopulationen zu transferieren. Diese Antigene umfassten adhäsive und chemotaktische Marker wie CD11b und CC-Chemokinrezeptor 2 (CCR2), Marker der Aktivierung und Differenzierung wie CD64, CD116 und CD132 und immunregulatorische Marker wie CD172a/b.

Diese Publikationspromotion dient daher der detaillierten Charakterisierung der phänotypischen und funktionellen Adaptation der Monozyten-Subpopulationen im Verlauf der gesunden Schwangerschaft bei Aktivierung, um ihren potenziellen Beitrag zur immunologischen Balance evaluieren zu können.

## 2.2. Material und Methoden

*Eine detailliertere Beschreibung der Materialien und Methodik inkl. der Herstellerangaben findet sich in der publizierten Originalarbeit (siehe S. 4ff. in dieser Dissertation).*

Als Grundlage der Experimente dienten Blutproben schwangerer Frauen aus der PRINCE (Prenatal Identification of Children‘s Health) Studie, einer populationsbasierten prospektiven Geburtenkohortenstudie des Universitätsklinikums Hamburg-Eppendorf (UKE). Eingeschlossen wurden volljährige, nicht-rauchende Schwangere mit Einlingsgravidität und natürlicher Konzeption. Die Ausschlusskriterien der hier untersuchten Untergruppe (n=15) umfassten die Einnahme immunsuppressiver Medikamente, chronische Erkrankungen, Schwangerschaftskomplikationen sowie -infektionen. Am Ende jedes Trimenons (Gestationswoche 13-15, 23-25, 35-37) erfolgte neben einer klinischen Untersuchung von Mutter und Kind die Gewinnung maternaler venöser Blutproben. Die Serumröhrchen wurden analog zu den Herstellervorgaben prozessiert, aus EDTA-Blut erfolgte die Gewinnung von mononukleären Zellen des peripheren Blutes (PBMC) mittels Dichtegradientenzentrifugation. Zur Hormonquantifizierung wurden die Serumproben, zur phänotypischen Analyse die PBMC von 15 Teilnehmerinnen nach passagerer Kryokonservierung retrospektiv analysiert. Für die Untersuchung der sekretorischen Monozytenfunktion wurden zudem frisch isolierte PBMC einer anderen Untergruppe der PRINCE Studie (n=21) prospektiv untersucht. Als alterskorrelierte Kontrollgruppe wurden nicht-schwangere Frauen und Männer innerhalb der Hamburger Gesundkohorte am UKE für eine einmalige Blutentnahme rekrutiert. Beide Studien (PV3694, PV4780) wurden von der Ethikkommission der Ärztekammer Hamburg genehmigt und jede teilnehmende Person unterzeichnete eine schriftliche Einwilligungserklärung vor dem Studieneinschluss.

Die Quantifizierung der Serumkonzentrationen der weiblichen Sexualhormone erfolgte am Zentrallabor des UKE gemäß den Herstellerangaben und den Standard Operating Procedures des Labors. Dabei wurden 17β-Östradiol, Progesteron und Gesamt-β-hCG (intaktes humanes Choriongonadotropin-Heterodimer und freie β-Untereinheit) mittels verschiedener Chemilumineszenz-Immunoassays bestimmt.

Nach der Implementation von drei durchflusszytometrischen Panels mit individuell optimierten Einstellungen des Durchflusszytometers (PMT-Spannungen und Kompensation) und deren Testung auf Reproduzierbarkeit erfolgte die Immunphänotypisierung aufgetauter und fluoreszenzmarkierter PBMC. Bei jedem experimentellen Durchlauf wurden die drei PBMC-Proben einer schwangeren Frau aus den drei Trimena mit jeweils einer alterskorrelierten weiblichen und männlichen Kontrolle untersucht. Die Datenerhebung erfolgte mit einem BD LSRFortessa Durchflusszytometer

innerhalb von drei Wochen und bis auf eine Ausnahme mit identischen, täglich angepassten Voreinstellungen.

Das sekretorische Potenzial der Monozyten wurde durch die durchflusszytometrische Messung der intrazellulären Tumornekrosefaktor (TNFα)-, Interleukin 12 (IL-12)- und Interleukin 6 (IL-6)-Konzentrationen in der Ausgangslage und nach Stimulation mit Lipopolysaccharid (LPS) oder CL097 quantifiziert. Dabei wurden PBMC binnen drei Stunden nach Blutentnahme aus EDTA-Blut isoliert und für 17 Stunden inkubiert. Die Datenerhebung erfolgte prospektiv im Verlauf der Schwangerschaften mit einem BD LSRFortessa Durchflusszytometer.

Zur Datenanalyse der durchflusszytometrischen Daten wurde die FlowJo Software v10.4.2 verwendet. Die graphische Darstellung der Daten erfolgte mittels der FlowJo Software v10.4.2 und GraphPad Prism 8.2.0. Zur statistischen Auswertung wurde GraphPad Prism 7.04 genutzt. Dabei wurde der gepaarte Vergleich von Zeitpunkten im Verlauf der Schwangerschaft mittels Wilcoxon-Vorzeichen-Rang-Tests durchgeführt, während der Mann-Whitney-U-Test der Ermittlung signifikanter Unterschiede zwischen Schwangeren eines bestimmten Trimenons und den nicht-schwangeren Kontrollprobandinnen diente. Die Multiplizität aller durchgeführten Tests wurde mittels des Benjamini-Hochberg-Verfahrens (Benjamini und Hochberg, 1995) adjustiert. Alle erwähnten p-Werte sind für die Falscherkennungsrate korrigiert, $p < 0,05$ wird als signifikant erachtet.

Die Hauptkomponentenanalyse wurde mit XLSTAT berechnet und graphisch dargestellt.

## 2.3. Ergebnisse

*Die im Folgenden erwähnten Abbildungen und Tabellen beziehen sich auf die Graphiken in der obigen Publikation inklusive des Anhangs (Anh.).*

### 2.3.1. Zusammensetzung und Aktivierung der Monozyten und ihrer Subpopulationen

Die Analyse und der Vergleich von Zusammensetzung und Phänotyp der Monozyten-Subpopulationen erfolgte trimenonweise in PBMC schwangerer Frauen mittels Durchflusszytometrie. Die detaillierten Teilnehmendencharakteristika dieser Analyse finden sich in Tabelle 1. Nach dem Ausschluss avitaler Zellen wurden Monozyten definiert als $lin^{neg}$ ($CD3^{neg}$, $CD20^{neg}$, $CD56^{neg}$), HLA-$DR^{pos}$ and $CD14^{pos}$. $CD56^{pos}$ Monozyten wurden separat analysiert (Daten nicht gezeigt). Ihr durchschnittlicher Anteil an den vitalen PBMC bewegte sich zwischen 0,13% und 0,17% während der Schwangerschaft und in der Kontrollgruppe. Die Einteilung in die drei Monozyten-Subpopulationen erfolgte unter Berücksichtigung der Nomenklatur von Monozyten und dendritischen Zellen des Blutes der International Union of Immunological Societies (Ziegler-Heitbrock et al., 2010) (Abb. 1A).

Es zeigte sich ein prozentualer Anstieg der $CD14^{pos}$ Gesamt-Monozyten innerhalb der vitalen PBMC zum Ende der Schwangerschaft hin (Abb. 1B), welcher mit einem signifikant steigenden Anteil der intermediären Subpopulation (Abb. 1D) einherging. Dieser steigende Anteil bestätigte frühere Beobachtungen (Melgert et al., 2012, Groen et al., 2015, Ziegler et al., 2018) einer signifikanten Verschiebung in der Zusammensetzung der Monozyten von klassischen hin zu intermediären Monozyten mit zunehmendem Gestationsalter (Abb. 1C und 1D), bei unverändertem Prozentsatz nicht-klassischer Monozyten (Abb 1E). Die Veränderungen des klassischen sowie des intermediären Monozytenanteils erwiesen sich auch im Vergleich mit nicht-schwangeren Frauen (Abb. 1C und 1D) als signifikant.

Um den Aktivierungsstatus der Monozyten während der Schwangerschaft zu bestimmen, wurden lösliche Serummarker, welche die Monozytenfunktion widerspiegeln, bestimmt. Im Verlauf der Schwangerschaft erhöhten sich die Spiegel der Aktivierungsmarker sCD163 und sCD14 signifikant, während die Spiegel des Chemokins CCL2 signifikant sanken (Anh. Abb. 1).

Zur Einschätzung von Ausmaß und Folgen der Aktivierung der einzelnen Monozyten-Subpopulationen erfolgte die durchflusszytometrische Analyse von Oberflächenantigenen, welche während der Schwangerschaftsphasen oder auf den Subpopulationen unterschiedlich exprimiert werden (Anh. Tabelle 2). Die Hauptkomponentenanalyse dieser Daten von den verschiedenen Monozyten-Subpopulationen offenbarte, dass nicht-schwangere und schwangere Frauen phänotypisch substanziell voneinander abweichen (Abb. 2A). Diese Unterschiede blieben konstant über die Gestation und veränderte Hormonspiegel hinweg. In der Projektion der Probandinnen gruppierten sich die Monozyten Schwangerer nicht nach dem jeweiligen Trimenon, sondern nach der individuellen

Probandin (Anh. Abb. 2). Außerdem separierten sich die intermediären von den nicht-klassischen Monozyten, wobei erstere über alle Trimena hinweg enger mit der Schwangerschaft in Beziehung standen (Abb. 2B, das dritte Trimenon zeigend). Insbesondere die Expression von CD132, CD14, CD64, CD11b und CD116 auf intermediären Monozyten, von CD32 auf klassischen Monozyten und die Toll-like-Rezeptor (TLR) 4 - Expression auf intermediären und nicht-klassischen Monozyten war mit der Schwangerschaft assoziiert (Abb. 2B, oberer rechter Quadrant).

Im Allgemeinen unterschieden sich die Monozyten-Subpopulationen beträchtlich hinsichtlich der Expression von Oberflächenantigenen für die Aktivierung und Differenzierung (Anh. Abb. 3). Zudem wich diese Expression innerhalb einer Subpopulation im Vergleich von Kontrollprobandinnen und -probanden teils signifikant voneinander ab, wie z.B. bei CD11b auf klassischen Monozyten oder bei CD11c (Daten nicht gezeigt). Das Ausmaß der Expression von CD11b, CD39, CD54, CD64 und CD116 auf allen $CD14^{pos}$ Monozyten war im dritten Trimenon verglichen mit dem ersten signifikant erhöht (Anh. Abb. 4), mit einem Trend progredient ansteigender medianer Fluoreszenzintensitäten (MFI) von CD11b, CD39, CD54 und CD64 im Laufe der Schwangerschaft. Die eingehende phänotypische Analyse der Monozyten-Subpopulationen offenbarte folgende Veränderungen: Die CD54-Expression nahm im Verlauf der Schwangerschaft lediglich auf der klassischen Monozyten-Subpopulation zu. CD14, CD132, CD172a/b und CCR2 stiegen auf der intermediären Monozyten-Subpopulation progredient im Verlauf der Schwangerschaft an. CD11b, CD64 und CD116 stiegen auf der klassischen und der intermediären Monozyten-Subpopulation. Im Phänotyp der nicht-klassischen Monozyten-Subpopulation manifestierten sich hingegen keine signifikanten Veränderungen (Abb. 2D, Anh. Tabelle 4). Zusammenfassend zeigen diese Daten, dass sich die schwangerschaftsassoziierte Aktivierung der Monozyten in veränderten Oberflächenantigenen für Aktivierung und Differenzierung widerspiegelt. Diese spezifische Adaptation ist auf der intermediären Monozyten-Subpopulation am stärksten ausgeprägt und ermöglicht eine Unterscheidung zwischen schwangeren und nicht-schwangeren Frauen.

### 2.3.2. Korrelation der schwangerschaftsassoziierten Sexualhormone zu Oberflächenantigenen auf den Monozyten-Subpopulationen

Um eine mögliche Kontribution der schwangerschaftsassoziierten Sexualhormone auf die Expression der signifikant veränderten Oberflächenantigene (Abb. 2D) zu eruieren, wurden zunächst die Serumspiegel von 17β-Östradiol, Progesteron und humanem Choriongonadotropin (β-hCG) im Verlauf der Schwangerschaft bestimmt (Abb. 3A). Die Korrelation dieser Hormone mit den veränderten Oberflächenantigenen offenbarte eine Assoziation von lediglich β-hCG mit dem Phänotyp der Monozyten-Subpopulationen (Abb.

3B, Anh. Tabelle 5). Im Detail zeigte sich eine signifikante negative Assoziation der β-hCG-Konzentration mit (i) der Expression von CD116 auf klassischen Monozyten im ersten Trimenon der Schwangerschaft, (ii) eine signifikante negative Assoziation mit der Expression von CD11b auf klassischen Monozyten im zweiten Trimenon der Schwangerschaft und (iii) eine signifikante positive Assoziation mit der Expression von CCR2 auf intermediären Monozyten im dritten Trimenon der Schwangerschaft (Abb. 3B). Um die Assoziation der veränderten Expression von CD116, CD11b und CCR2 mit der Schwangerschaft darüber hinaus zu verifizieren, wurde die Expression dieser Oberflächenantigene auf den Monozyten-Subpopulation zwischen schwangeren und nicht-schwangeren Frauen verglichen (Abb. 3C). Es gab einen signifikanten Unterschied in der Expression von CD116, CD11b und CCR2 auf intermediären Monozyten zwischen schwangeren und nicht-schwangeren Frauen im dritten (CD116) oder im zweiten und dritten Trimenon der Schwangerschaft (CD11b, CCR2) (Abb. 3C). Resümierend verdeutlichen diese Daten, dass die Adaptationen im Phänotyp der Monozyten-Subpopulationen im Verlauf der Schwangerschaft mit den β-hCG-Spiegeln assoziiert sind.

### 2.3.3. Sekretorisches Potenzial der Monozyten

Um die Einflüsse von Monozyten auf inflammatorische Immunantworten bei Schwangeren zu beleuchten, wurde das sekretorische Potenzial zirkulierender Monozyten mittels intrazellulärer Zytokinmessung nach Inkubation mit LPS oder CL097 bestimmt. Quantifiziert wurde der Anteil der Monozyten, welcher TNFα, IL-12 oder IL-6 produzierte.

Verglichen mit Schwangeren im ersten Trimenon wiesen Monozyten von Schwangeren im dritten Trimenon niedrigere basale IL-6-Level auf (Abb. 4A). Zudem zeigte sich zum Ende der Schwangerschaft hin eine signifikante Abnahme des Anteils $TNF\alpha^{pos}$ Monozyten nach LPS- oder CL097-Stimulation sowie des Anteils $IL\text{-}6^{pos}$ Monozyten nach LPS-Stimulation (Abb. 4A). Im Gegensatz dazu sank der Anteil $IL\text{-}12^{pos}$ Monozyten nach LPS- oder CL097-Stimulation im zweiten Trimenon, um im dritten Trimenon der Schwangerschaft wieder signifikant anzusteigen (Abb. 4A). Basierend auf der TNFα-, IL-12- und IL-6-Produktion in den drei Trimena wurde im Anschluss die simultane Produktion mehrerer Zytokine (Polyzytokinproduktion) in den einzelnen, inkubierten Monozyten mittels Boolean Gating bestimmt. Insgesamt nahm die Polyzytokinproduktion der Monozyten nach Stimulation zum Ende der Schwangerschaft hin ab, während die Anzahl der Monozyten, die mit der Produktion maximal eines Zytokins auf die Stimulation reagierten, im Verlauf der Schwangerschaft anstieg (Abb. 4B, Kreisdiagramme). IL-6, jedoch nicht TNFα und IL-12, schien im dritten Trimenon der Schwangerschaft an Bedeutung zu gewinnen (Abb. 4B). Zusammenfassend verändert sich das sekretorische Potenzial stimulierter Monozyten während gesunder humaner Schwangerschaften mit einer spezifischen Reduktion der Polyzytokin-Antworten.

## 2.4. Diskussion

Die Eigenschaften der Monozyten tragen zur Aufrechterhaltung der immunologischen Balance im Laufe der Schwangerschaft bei. In dieser Dissertation offenbarte die phänotypische Analyse der Adaptation der Monozyten-Subpopulationen im Verlauf der gesunden Schwangerschaft, dass die Verschiebung in der Monozytenzusammensetzung begleitet wird von einer signifikant höheren Expression von Oberflächenantigenen der Differenzierung, Adhäsion und Aktivierung nach Stimulation mit Pathogen-assoziierten molekularen Mustern (PAMPs), humoralen und zellulären Signalen. Diese Hochregulierung war auf der intermediären Monozyten-Subpopulation am ausgeprägtesten und konnte in Teilen mit den β-hCG-Serumspiegeln assoziiert werden. Funktionell zeigte sich eine gesteigerte monozytäre IL-12-Produktion nach Stimulation im dritten Trimenon, wohingegen die basale und stimulierte TNFα- und IL-6-Produktion sowie das polysekretorische Potenzial der Gesamt-Monozyten progredient abnahmen.

Es wird angenommen, dass klassische Monozyten sequenziell in intermediäre und nicht-klassische Monozyten übergehen (Patel et al., 2017), unter progredienter Zunahme des Reifegrads (Ancuta et al., 2009). Im Verlauf der Schwangerschaft schrumpft der Anteil klassischer Monozyten signifikant zugunsten einer steigenden Fraktion intermediärer Monozyten (Melgert et al., 2012, Groen et al., 2015, Ziegler et al., 2018). Vergleichbar nimmt die intermediäre Monozyten-Subpopulation auch bei Infektionserkrankungen mit dem Humanen Immundefizienz-Virus Typ 1 (HIV-1), dem Zika-Virus oder kongenitaler Toxoplasmose sowie bei (auto-) entzündlichen Erkrankungen zu (Wacleche et al., 2018, Michlmayr et al., 2017, Machado et al., 2014, Gren et al., 2015). Dies legt ein proinflammatorisches Milieu als Treiber der Expansion intermediärer Monozyten nahe. Während der Schwangerschaft wird die Verminderung der klassischen Monozyten-Subpopulation zudem auf eine Rekrutierung an die fetomaternale Grenzzone zurückgeführt, mit einer kompensatorisch erhöhten Aktivierung der verbleibenden Monozyten zur Gewährleistung einer effektiven Infektabwehr (Svensson-Arvelund et al., 2014). In diesem Kontext wurden der intermediären Monozyten-Subpopulation immunregulatorische Funktionen durch die Interaktion mit regulatorischen T-Zellen zugeschrieben (Faas und de Vos, 2017). Daher könnte der gestiegene Anteil intermediärer Monozyten zu einer präzisen Anpassung immunologischer Antworten während der Schwangerschaft beitragen.

Die detaillierte phänotypische Charakterisierung der drei Monozyten-Subpopulationen in dieser Dissertation offenbarte einige durch die Schwangerschaft erheblich veränderte Oberflächenantigene, was sich in deren Assoziation mit dem Glykoproteinhormon β-hCG und in der abweichenden Expression bei schwangeren und nicht-schwangeren Frauen widerspiegelte. Darunter befanden sich CCR2, CD11b und CD116. Interessanterweise

unterschied sich die Expression dieser Oberflächenantigene während des ersten Trimenons nicht signifikant von den nicht-schwangeren Frauen. Dennoch war bei Schwangeren im ersten Trimenon, in welchem die β-hCG-Spiegel ihr Maximum erreichen, die Oberflächenexpression von CD116 auf klassischen Monozyten signifikant negativ mit den β-hCG-Konzentrationen assoziiert. CD116 (Granulozyten-Makrophagen-Kolonie-stimulierender Faktor-Rezeptor GM-CSFRα) wurde trotz seiner Schlüsselrolle in der Monozytendifferenzierung und -funktion bisher nicht eingehend auf Monozyten gesunder Schwangerer charakterisiert. Hier zeigte sich ein Anstieg der CD116-Expression auf klassischen und intermediären Monozyten zum Ende der Schwangerschaft hin. Dieser Anstieg, gemeinsam mit den fehlenden Veränderungen im ersten Trimenon und der Assoziation zu den β-hCG-Spiegeln, reflektiert die immunologischen Phasen der Schwangerschaft. Das erste Trimenon wird als inflammatorischer Zustand erachtet (Mor und Cardenas, 2010) und könnte die Verminderung der Monozytendifferenzierung und ihrer Effektorfunktionen zur Reduktion entweder regulatorischer oder überschießender inflammatorischer Immunantworten erfordern. Vor dem Hintergrund des proinflammatorischen sekretorischen Potenzials im ersten Trimenon erscheint Letzteres wahrscheinlicher. CD11b ist häufig in der Schwangerschaft mit widersprüchlichen Resultaten untersucht worden (Sacks et al., 1998, Luppi et al., 2002a, Luppi et al., 2002b, Mikhaylova et al., 2013). Es wird angenommen, dass die eindrücklichste Hochregulierung dieses Adhäsionsmoleküls mit dem Beginn der Geburt assoziiert ist und auf eine weitere Monozytenaktivierung hindeutet (Luppi et al., 2002a). Im Gegensatz dazu konnten andere Autoren keine signifikanten Veränderungen von CD11b während der Schwangerschaft feststellen (Zhang et al., 2017). Ähnlich wie bei CD116 identifizierte diese Arbeit einen Anstieg von CD11b auf klassischen und intermediären Monozyten zum Ende der Schwangerschaft hin sowie eine starke negative Assoziation mit β-hCG im zweiten Trimenon. Unter Berücksichtigung der Hochregulierung weiterer Antigene für Aktivierung, Differenzierung und Adhäsion erscheint eine gesteigerte Monozytenextravasation mit nachfolgender Differenzierung in Monozyten-abgeleitete dendritische Zellen und Makrophagen mit der Progression der Schwangerschaft wahrscheinlich. Dieser Prozess könnte durch einen im Laufe der Schwangerschaft progredienten Anstieg von GM-CSF (Vassiliadis et al., 1998), dem Liganden von CD116, gefördert werden. Abermals erscheint eine präzise adjustierte Balance essenziell, da erhöhte Mengen von GM-CSF und Makrophagen in präeklamptischer Dezidua vorgefunden worden sind (Huang et al., 2010) und bei präeklamptischen Schwangerschaften von einer übermäßigen CD11b-Expression auf Monozyten berichtet worden ist (Faas und de Vos, 2017).

CCR2 rekrutiert Monozyten in entzündetes Gewebe. Publikationen zur monozytären CCR2-Expression während der Schwangerschaft demonstrierten eine Verminderung (Al-ofi et al.,

2012, Mikhaylova et al., 2013) sowie bei der termingerechten Geburt einen signifikanten Anstieg verglichen mit den ersten beiden Trimena (Zhang et al., 2017). Der Ligand CCL2 wird von humanen Deziduazellen in hohem Maße synthetisiert und seine Sekretion durch Progesteron sowie hohe β-hCG-Spiegel gesteigert (Caballero-Campo et al., 2002, He et al., 2007). Hier wurde eine progrediente Abnahme der CCL2-Serumspiegel im dritten Trimenon detektiert, welche mit einer signifikant gestiegenen CCR2-Expression auf intermediären Monozyten einherging. Die CCR2-Expression war zudem positiv mit der β-hCG-Serumkonzentration im dritten Trimenon assoziiert. HCG kann mit dem LH-, FSH- und TSH-Rezeptor interagieren (Napso et al., 2018), jedoch ist die hCG-Signaltransduktion über diese Rezeptoren auf Monozyten kontrovers. Weiterhin werden nicht-kanonische hCG-Rezeptoren auf Immunzellen diskutiert, beispielsweise TLR4 (Zamorina und Shirshev, 2014), C-Typ Lektine (Kosaka et al., 2002) oder der Mannoserezeptor CD206 (Kane et al., 2009). Eine *in vitro* Exposition von PBMC oder myeloiden THP-1-Zellen mit unterschiedlichen β-hCG-Konzentrationen bewirkte keine phänotypischen Veränderungen innerhalb der Monozytenpopulation, sodass kein direkter Effekt von β-hCG auf Monozyten anzunehmen ist (Daten nicht gezeigt). Weitere Studien sind vonnöten zur Testung, ob der Einfluss von β-hCG auf humane Deziduazellen zu einer Attraktion CCR2-exprimierender intermediärer Monozyten in verschiedenen Schwangerschaftsstadien führt, möglicherweise durch die Steigerung der CCL2-Expression in der Dezidua und einem dadurch bedingten erhöhten Chemokingradienten.

Neben den phänotypischen Veränderungen der Monozyten im Laufe der Schwangerschaft ist insbesondere deren funktionelle Adaptation relevant. Diese stellt sich in Studien uneinheitlich dar (Faas und de Vos, 2017). Es wird vornehmlich davon ausgegangen, dass die Aktivierung der Monozyten während der Schwangerschaft zu einer erhöhten LPS-Toleranz führt (Faas et al., 2002), mit dem Resultat reduzierter Zytokinantworten. Damit übereinstimmend wurde in dieser Arbeit eine verminderte IL-6- und TNFα-Produktion gegen Ende der Schwangerschaft beobachtet, obwohl die intermediäre Monozyten-Subpopulation mit proinflammatorischer Funktion während der Schwangerschaft größer wird. Sowohl erhöhte IL-6- als auch TNFα-Spiegel im Fruchtwasser oder Scheidensekret wurden als Prädiktoren einer Frühgeburt beschrieben (Yockey und Iwasaki, 2018) und erfordern daher eine strikte Regulierung zum Ende einer gesunden Schwangerschaft hin. Die verminderte IL-12-Produktion lediglich im zweiten Trimenon entspricht dem natürlichen immunologischen Verlauf der Schwangerschaft, mit proinflammatorischem Milieu im ersten Trimenon und peripartal im Gegensatz zu einer antiinflammatorischen Umgebung im zweiten Trimenon während des fetalen Wachstums (Mor und Cardenas, 2010). TLR7 und -8 sind an frühen Reaktionen des angeborenen Immunsystems auf relevante Gestationsinfektionen mit einzelsträngigen RNA-Viren beteiligt, wie beispielsweise

Influenza-, Zika- oder Rubella-Viren, welche ungünstige Folgen auf den regelrechten Verlauf der Schwangerschaft bei Mutter und Kind ausüben können (Goldenberg et al., 2000, Racicot und Mor, 2017). Die gesteigerte IL-12-Produktion nach TLR7/8-Stimulation mittels CL097 im dritten Trimenon der Schwangerschaft spiegelt mutmaßlich ein gesteigertes antivirales Potenzial wider, da IL-12 die Th1-Differenzierung und Th1-Effektorfunktionen induziert (Ramshaw et al., 1997). Des Weiteren legt diese Dissertation nahe, dass Monozyten mit spezifischen Funktionen im letzten Trimenon einen wichtigen Stellenwert einnehmen. Insgesamt zeigten Monozyten eine Verminderung ihres polysekretorischen Potenzials zum Ende der Schwangerschaft hin. Da eine proinflammatorische Signaltransduktion zur Geburtsinduktion beiträgt und insbesondere steigende IL-6-Spiegel eine wichtige Rolle zu spielen scheinen (Peltier, 2003), könnte der gestiegene Anteil ausschließlich IL-6 produzierender Monozyten nach LPS-Stimulation im dritten Trimenon zu diesem Prozess beitragen.

Auf gesunden Schwangerschaften mit gesundem Nachwuchs lag bisher kein großer Forschungsschwerpunkt. Untersuchungen des maternalen Immunsystems während physiologischer Schwangerschaften werden das Verständnis pathophysiologischer Reaktionen während der Gestation verbessern und schaffen eine Grundlage, um Schwangerschaften mit widrigem Ausgang für Mutter und Kind mit dem maternalen Immunsystem während der Schwangerschaft in Verbindung zu setzen. Diese Dissertation beschreibt als erste die detaillierten phänotypischen Veränderungen auf definierten Monozyten-Subpopulationen in jedem Trimenon und die Assoziation dieser Erkenntnisse mit den β-hCG-Spiegeln gesunder Schwangerer. Darüber hinaus wurden die spezifischen Adaptationen des sekretorischen Potenzials von Monozyten an die Schwangerschaft beleuchtet. Diese Dissertation stellt daher wichtige Referenzdaten zu Phänotyp und Funktion der Monozyten-Subpopulationen in jedem Stadium einer gesunden humanen Schwangerschaft zur Verfügung und ermöglicht zukünftige Vergleiche mit Schwangerschaften mit widrigem Ausgang, einschließlich der Präeklampsie.

# 3. Zusammenfassung

## 3.1. Zusammenfassung in englischer Sprache

This doctoral thesis provides a comprehensive, longitudinal overview of phenotypic alterations and secretory potential of monocytes in healthy pregnant women.

Flow cytometry was performed using blood samples from each trimester of pregnancy for in-depth characterization of monocyte subsets, and confirmed a pregnancy-induced, gradual shift from classical towards intermediate monocytes. Principal component analysis revealed specific phenotypic changes in monocyte subsets throughout pregnancy, which were most pronounced in the intermediate subset and especially occurred in surface markers involved in activation, differentiation and adhesion. Pregnancy-related hormones were quantified in serum and a significant association between human chorionic gonadotropin levels and the expression of CD11b, CD116 and CCR2 on monocyte subsets was found. Stimulation of bulk monocytes via TLR4 and TLR7/8 pathway resulted in diminished polycytokine production towards the end of pregnancy.

These results elaborate a specific contribution of distinct monocyte subsets to healthy pregnancy and therefore serve as reference data for future comparisons and evaluation of pregnancies with adverse outcomes.

## 3.2. Zusammenfassung in deutscher Sprache

Diese Doktorarbeit bietet einen umfassenden, longitudinalen Überblick der phänotypischen Veränderungen und des sekretorischen Potenzials von Monozyten gesunder Schwangerer.

Die Durchflusszytometrie von Blutproben aus jedem Trimenon der Schwangerschaft wurde zur eingehenden Charakterisierung der Monozyten-Subpopulationen durchgeführt und bestätigte die schwangerschaftsinduzierte, sukzessive Verschiebung von klassischen hin zu intermediären Monozyten. Die Hauptkomponentenanalyse offenbarte spezifische phänotypische Veränderungen der Monozyten-Subpopulationen, die in der intermediären Subpopulation am ausgeprägtesten waren und insbesondere Oberflächenmarker für Aktivierung, Differenzierung und Adhäsion betrafen. Schwangerschaftsassoziierte Hormone wurden im Serum quantifiziert und es zeigte sich eine signifikante Assoziation der humanen Choriongonadotropin-Spiegel mit der Expression von CD11b, CD116 und CCR2 auf verschiedenen Monozyten-Subpopulationen. Die Stimulation der Gesamt-Monozyten über den TLR4- und TLR7/8-Signalweg resultierte zum Ende der Schwangerschaft hin in einer verminderten Polyzytokinproduktion.

Diese Ergebnisse erarbeiten den spezifischen Beitrag unterschiedlicher Monozyten-Subpopulationen zu einer gesunden Schwangerschaft und dienen daher als Grundlage für Vergleiche mit und die Evaluation von Schwangerschaften mit widrigem Ausgang.

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**Erklärung des Eigenanteils**

Die nachstehend aufgeführten Arbeiten für die vorliegende Publikationspromotion wurden von mir, Caroline Pflitsch, **selbstständig oder in Zusammenarbeit** durchgeführt:

- Verarbeitung der Blutproben der Hamburger Gesundkohorte
- Erstellung, Testung und Optimierung der durchflusszytometrischen Panels (mit Dr. S. Ziegler, Prof. Dr. M. Altfeld)
- Erhebung und Analyse der durchflusszytometrischen Daten zur phänotypischen Charakterisierung
- Quantifizierung der löslichen Serummarker (mit Dr. S. Ziegler)
- Statistische Auswertung (mit Dr. L. Richert, Dr. S. Ziegler)
- Erstellung der Abbildungen (mit Dr. S. Ziegler, Dr. L. Richert)
- Literaturrecherche und -auswertung
- Schreiben des Manuskripts (mit Dr. S. Ziegler)
- Verfassen dieser Publikationspromotion

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## 6. Lebenslauf

Lebenslauf wurde aus datenschutzrechtlichen Gründen entfernt.

## 7. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
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