



Research paper

The influence of maternal obesity on macrophage subsets in the human decidua

A. Laskewitz^{a,b,1}, K.L. van Benthem^{a,1}, T.E.C. Kieffer^a, M.M. Faas^{a,b}, R.N. Verkaik-Schakel^a, T. Plösch^a, S.A. Scherjon^a, J.R. Prins^{a,*}

^a Department of Obstetrics and Gynecology, University of Groningen and University Medical Center Groningen, PO Box 30001, 9700 RB Groningen, The Netherlands

^b Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, PO Box 30001, 9700 RB Groningen, The Netherlands

ARTICLE INFO

Keywords:

Macrophages
Decidua
Obesity
Pregnancy complications
Placenta
Inflammation

ABSTRACT

Obesity is seen as a low grade inflammatory state, and is associated with adverse pregnancy outcomes. Disturbed macrophage characteristics might be essential in obesity associated pregnancy pathology via effects on the regulation of angiogenesis and placental development. This study aims to address the effects of maternal obesity on macrophage subsets in the decidua of women with term uncomplicated pregnancies. Macrophages were isolated from the decidua basalis and decidua parietalis of women with pre-gravid BMI < 25 (control) and BMI > 30 (obese). Macrophages were characterized and quantified using multi-color flow cytometry. Placentas of 10 obese and 10 control women after an uncomplicated term pregnancy were included. The decidua parietalis, but not decidua basalis, showed significantly lower levels of M1-type (HLA-DR⁺, CD163⁻) macrophages ($p < 0.05$) in obese women (4,3% of total macrophages) compared to control women (5,3% of total macrophages). The lower levels of M1 macrophages, considered to be pro-inflammatory, might indicate a mechanism to compensate for the pro-inflammatory environment in obese women to ensure healthy pregnancy outcomes.

1. Introduction

Obesity in women of reproductive age constitutes a growing health problem throughout the world [1,2]. Obesity is classified as a body mass index (BMI) of $\geq 30 \text{ kg/m}^2$ [3,4]. In general, obesity shows characteristics of an inflammatory state, such as raised CRP, a lower serum albumin, and higher leucocytes in the peripheral blood [3,5]. During pregnancy, obesity is associated with various unfavorable pregnancy outcomes, such as: spontaneous miscarriage, congenital anomalies, gestational diabetes, preeclampsia, and fetal macrosomia [1,3,6–10]. Thus far, the biological mechanisms behind the association between obesity and unfavorable pregnancy outcomes are only partly understood. Possibly, a disturbed maternal immune balance, such as altered macrophage characteristics are involved in the complicated pregnancy outcomes.

Macrophages constitute a major and stable leukocyte population in the decidua. The maternal term decidua consists of distinct regions, the decidua basalis and decidua parietalis. Decidual macrophages have diverse functions during pregnancy such as protection against

pathogens, and are important in the normal development of the placenta [5,11]. Decidual macrophages are also involved in maternal immunoregulation towards the semi-allogeneic fetus [6,11–14]. These functionally different roles are attributed to distinct macrophage subsets [15,16]. Classically two distinct macrophage subsets are described, based on their function and phenotype; the pro-inflammatory M1 subset and the anti-inflammatory M2 subset. M1 macrophage activation is induced by inflammatory factors, such as LPS activation, IFN- γ , and TNF- α , resulting in the production of inducible nitric oxide synthase (iNOS) and promotion of T-helper 1 responses [16,17]. Markers of M1 macrophages are IRF-5, HLA-DR, and CD80⁺ [15–19]. M2 macrophages are associated with maternal tolerance to the fetus and exhibit immunoregulatory actions by production of anti-inflammatory IL-10, IL-1 receptor antagonist, TGF- β and the enzyme arginase [20–23]. M2 macrophages in the placental bed might be slightly different from systemic M2 macrophages since in *in vitro* studies they have been shown to be induced by M-CSF and IL-10 [11,24]. Macrophage mannose receptor C type (MRC), CD206⁺, CD163⁺, and CCL18 are markers of M2 polarization [18,25–27]. Uterine macrophages are characterized by

* Corresponding author.

E-mail addresses: a.laskewitz@umcg.nl (A. Laskewitz), k.l.van.benthem@umcg.nl (K.L. van Benthem), t.e.c.kieffer@umcg.nl (T.E.C. Kieffer), m.m.faas@umcg.nl (M.M. Faas), r.n.schakel@umcg.nl (R.N. Verkaik-Schakel), t.plosch@umcg.nl (T. Plösch), s.a.scherjon@umcg.nl (S.A. Scherjon), j.r.prins@umcg.nl (J.R. Prins).

¹ Contributed equally.

<https://doi.org/10.1016/j.cellimm.2019.01.002>

Received 30 May 2018; Received in revised form 15 December 2018; Accepted 10 January 2019

Available online 11 January 2019

0008-8749/© 2019 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

their phenotypic plasticity, switching between M1 and M2 phenotypes, to adjust to a variety of distinctive environments, most probably also induced by fetal antigens [18,26,28]. The general classic separation in pro-inflammatory M1 and anti-inflammatory M2 macrophage subsets appears less strict as there are overlapping phenotypes [29].

Decidual macrophages differ in their characteristics and functioning depending on gestational age. Reports demonstrate increased levels of M2 decidual macrophages in the second and third trimester of pregnancy compared to the first trimester [5,28,30]. M1 maternal macrophages infiltrate the decidua at the end of the third trimester, developing a pro-inflammatory state associated with the onset of labor [18,31]. Decidual M1/M2 imbalances are implicated into a spectrum of pregnancy complications as miscarriage, preeclampsia, and intrauterine infections [3,27,30,32]. Obesity is known to result in a low-grade inflammatory state and placental inflammation [3,5,26,33,34]. Abnormal macrophage function in the placenta might be implicated in complicated pregnancy outcomes associated with obesity [3,35]. However, the precise effects of obesity on decidual macrophages are unclear. Possible compensatory mechanisms to ensure a healthy pregnancy outcome in obese women with a pro-inflammatory state are not known. This study aims to investigate the effects of pre-gravid maternal obesity on macrophage subsets in decidual tissues of uncomplicated term pregnancies in control and obese women.

2. Methods

2.1. Study design

In this study 20 human placentas were included. All placentas were delivered after term, singleton, uncomplicated pregnancies, at the Department of Gynecology and Obstetrics in the University Medical Center Groningen (UMCG), the Netherlands. All women reported no smoking, drugs, or alcohol use. To be included, the pre-gravid BMI of the pregnant women given birth had to be < 25 for the control group or > 30 for the obese group. Placentas were collected in phosphate buffered saline (PBS) within 60 min after birth, and stored (< 7 °C) for no longer than 24 h until further analysis [36]. Placental tissue is regarded as part of medical ‘rest material’ and therefore covered by the ‘code of conduct for responsible use’ according the Federation of Medical Scientific Associations (FEDERA) [37]. This study was approved by the Medical Ethics Review Board of the UMCG (number METc2018/516).

2.2. Isolation

The amniotic layer of the membranes was separated from the chorionic layer and decidua parietalis by gentle manual traction. Thereafter, a cell scraper was used to separate the decidua parietalis from the chorionic layer [12,38]. From the maternal surface of the placenta, pieces of 5 cm² in all quadrants of the decidua basalis were removed using scissors. Fetal villous tissue was removed as much as possible. To minimize cell death, all tissues layers were constantly embedded in PBS. Infarcted and discolored parts of tissue were not included and blood clots and vessels were removed.

After separation, both decidua parietalis and decidua basalis were directly placed in PBS, cut into small pieces using a scissor, washed extensively in PBS, and centrifuged at 210 × g at room temperature. Tissue was digested by the addition of StemPro Accutase Gibco (Thermo Fisher Scientific, Waltham, MA, USA) in a 1:2 ratio (tissue to accutase). The tissue was mixed and dissociated by a GentleMacs Dissociator (Miltenyi Biotech). For the decidua basalis a program of 17 s with 668 total rounds per run was used and the decidua parietalis ran twice for 37 s with 235 rounds per run. Tissue was incubated and gently shaken (~80/min) at 37 °C for 45 min. Hereafter, 10 mL of PBS was added to stop the digestive process, followed by filtration through a 70 µm Easy Strainer (Falcon). Thereafter, cells were washed in PBS, and

a Percoll (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) gradient was used to separate the cells based on their density. The macrophage enriched cell layer was isolated from the interface between densities of 1.023–1.053 g/mL.

2.2.1. Cell count

To determine the number of macrophages, a Beckman coulter counter (Beckman Coulter Life Sciences, Indianapolis, IN) was used. Macrophages were placed in 750 µL Roswell Park Memorial Institute Medium (RPMI) 1640 Gibco (Life Technologies, Carlsbad, CA, USA). Fetal calf Serum (FCS) (Greiner, Kremsmünster, Austria) was diluted with dimethylsulfoxide (DMSO) (Merck, Burlington, MA, USA) in a 4:1 ratio respectively and was added to the cell suspension in a 1:1 ratio and stored in liquid nitrogen (–180 °C) until analysis.

2.3. Flow cytometry

In order to thaw cells, cells frozen in liquid nitrogen were immediately placed in a 37 °C water bath and swirled until a small piece of ice was left in the vial. 4 mL of pre-warmed (37 °C) RPMI was applied dropwise, the cell suspension was centrifuged, and the cell pellet was resuspended into RPMI.

The cell suspensions were added to a 96-wells plate (Corning, New-York, USA) and centrifuged, the RPMI supernatant was discarded, and cells were incubated in the dark for 10 min with 50 µL Fixable Viability Stain (FVS) 620 (BD-Biosciences, Franklin Lakes, NJ, USA). To block nonspecific binding of Fc receptors 50 µL 1% Fc block (BD-Biosciences, Franklin Lakes, NJ, USA) with 10% mouse serum (Sanquin, Amsterdam, the Netherlands) was added for 10 min in the dark. Cells were incubated with a dilution of the (extracellular) antibody mix for 30 min at 4 °C in the dark (Table 1). To preserve the staining, cells were fixed using 200 µL Fixsolution (BD-Biosciences, Franklin Lakes, NJ, USA) for 40 min. FACS analysis was performed on a FACSVerse™ flow cytometer, (Becton-Dickinson and Company, Franklin Lakes, NJ, USA). Beads were stained with a single monoclonal antibody to set compensation (BD-Biosciences, Franklin Lakes, NJ, USA). To allow compensation of FVS, single stains were used (BD-Biosciences, Franklin Lakes, NJ, USA). Gating strategy was based on negative controls and positive populations (Fig. 1), and macrophage subsets (M1 and M2) and decidual macrophages (CD50⁺) were characterized based on recent studies [16,39]. Data analysis was done using FlowJo v10 software. Due to the low amount of cells that could be isolated, no fluorescence minus one stains could be performed.

2.4. Statistical analysis

To determine whether levels of macrophage subsets differed statistically significant between obese women and controls (i.e., BMI > 30 versus BMI < 25), and between the decidua parietalis and decidua basalis a Mann-Whitney *U* test was used.

For the statistical analyses GraphPad Prism version 7.03 for Windows 10 (GraphPad Software, CA, USA) was used. For all statistical tests, a *p*-value of < 0.05 was considered significant.

Table 1
Macrophage antibody mix.

Antibody	Conjugate	Clone	Dilution
FVS 620	PerCP-Cy5.5		1:50
CD45	APC-H7	2D1	1:20
CD14	BV510	MφP9	1:20
CD50 (ICAM3)	PE	TU41	1:50
HLA-DR	APC	G46-6	1:10
CD163	BV421	GHI/61	1:50

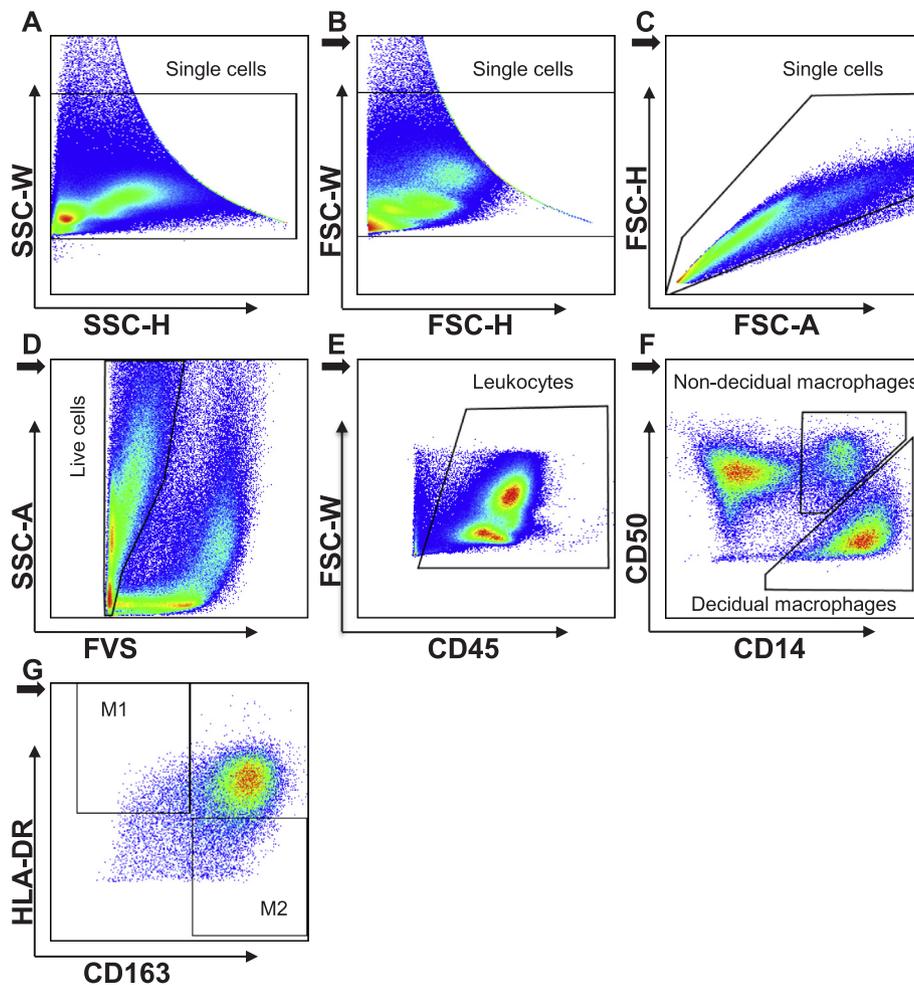


Fig. 1. Gating strategy to identify M1 and M2 macrophages. Single cells and living cells were gated using SSC and FSC (A-D). In the living cells leukocytes were identified (CD45⁺) (E) and within the leukocytes population the decidual macrophages (CD50⁻CD14⁺) and non-decidual macrophages (CD50⁺CD14⁺) were distinguished. Differentiation between M1 (HLA-DR⁺CD163⁻) and M2 macrophages (HLA-DR⁻CD163⁺) was done in HLA-DR vs. CD163 dotplots.

3. Results

3.1. Characteristics of study population

In this study macrophages were isolated from decidual tissue of 20 pregnancies. Table 2 presents patient characteristics. Our control group had a mean BMI of 21.1 kg/m², whereas our obese group had a mean BMI of 35.6 kg/m². Apart from BMI ($p < 0.0001$), no differences in

Table 2
Clinical characteristics of the study population.

	Control group BMI < 25 (n = 10)	Obese group BMI > 30 (n = 10)
BMI (kg/m ²)	21.1 ± 1.6	35.6 ± 3.6****
Age mother (years)	30.3 ± 4.4	28.2 ± 4.8
Gestational age at delivery (weeks)	40.2 ± 1.1	39.8 ± 0.9
Birth weight baby (gram)	3566 ± 615	3511 ± 480
Time between birth and isolation (hours)	10.3 ± 8.3	14.2 ± 7.6
Parity	1.5 ± 0.7	2.4 ± 1.5
Fetal sex; boy/girl	5/5	6/4
Mode of delivery; vaginal/secondary caesarean	9/1	10/0

Data were described as mean ± standard deviation and percentage for categorical variables.

BMI Body mass index. **** $p < 0.0001$ compared to control using Mann-Whitney U test.

patient characteristics were found between the two groups.

3.2. Lower levels of M1 macrophages in the decidua parietalis of obese women compared to control women

Using FACS analysis, no differences in total macrophages (CD14⁺CD45⁺) were found between the obese and control women in the decidua parietalis (Fig. 2B). However, percentages of pro-inflammatory M1 (HLA-DR⁺CD163⁻) macrophages were lower in the total macrophage population (CD14⁺CD45⁺; $p < 0.05$; Fig. 2C, Table 3), and in the decidual macrophage population (CD50⁻CD14⁺CD45⁺; $p < 0.01$; Fig. 2E, Table 3) in obese women compared to control women. Moreover, the geometric Mean Fluorescence Intensity (geo MFI) of the M1 marker HLA-DR showed a trend ($p = 0.06$) to be lower in obese women (Fig. 2F, Table 3). No differences were found for the levels of M1 in the non-decidual macrophage population and also not in the levels of M2 (HLA-DR⁻CD163⁺) macrophages in decidua parietalis between obese and control women (Table 3).

3.3. Comparable levels of M1 macrophages in the decidua basalis of obese and control women

Different to the macrophages in the decidua parietalis, there was a difference in total macrophage levels between the obese and control women, with lower levels of macrophages within the leukocytes in the

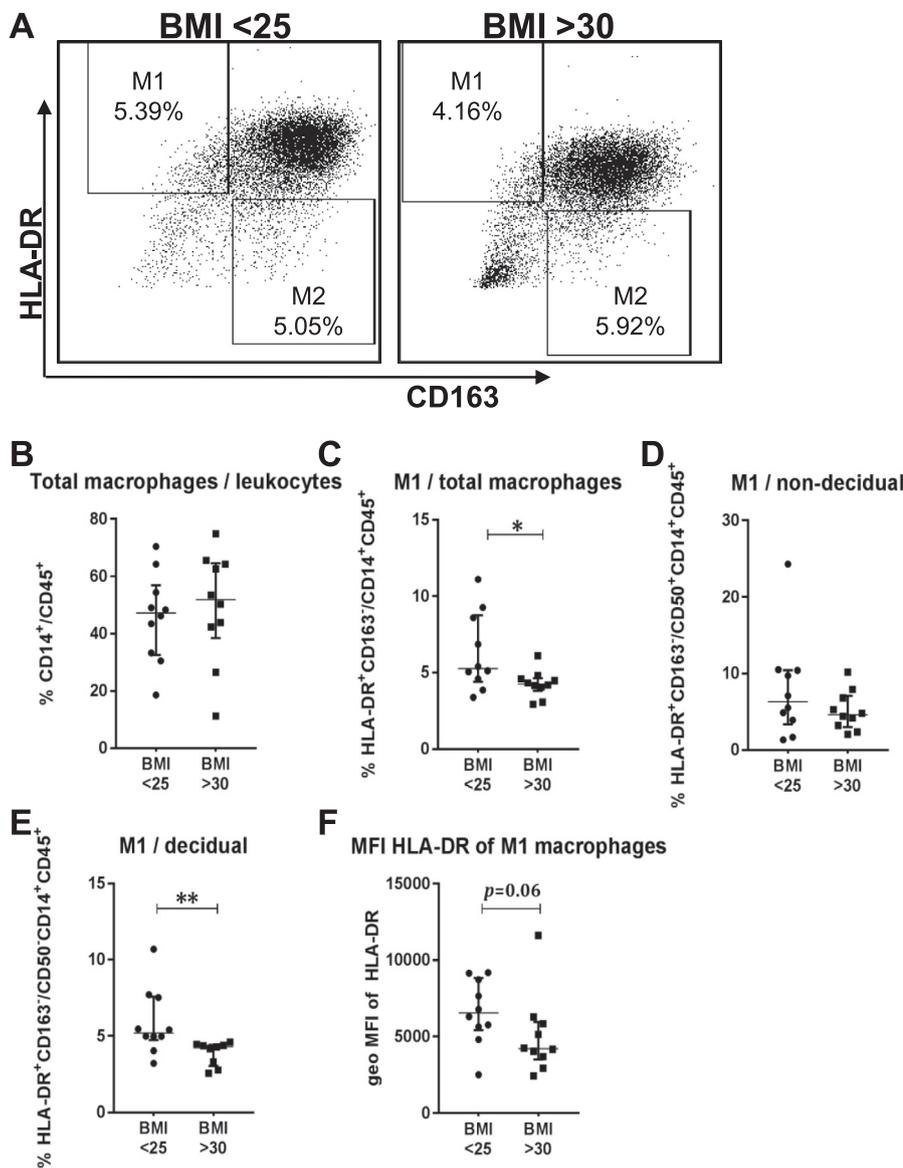


Fig. 2. Macrophages in decidua parietalis of women with a BMI < 25 kg/m² or with a BMI > 30 kg/m². Gating of M1 macrophages (HLA-DR⁺CD163⁻) and M2 macrophages (HLA-DR⁻CD163⁺) in different groups (A). Proportions of total macrophages (CD14⁺) of leukocytes (CD45⁺) (B), proportions of M1 macrophages (HLA-DR⁺CD163⁻) of total macrophages (C), proportions of M1 macrophages of non-decidual macrophages (CD50⁺CD14⁺CD45⁺) (D), and proportions of M1 macrophages of decidual macrophages (CD50⁻CD14⁺CD45⁺) (E). Geometric mean fluorescence intensity (geo MFI) of M1 macrophages of total macrophages (F). Symbols represent values of individual decidua. Horizontal lines are medians ± interquartile range. Analysis by Mann-Whitney U test; * = *p* < 0.05, ** = *p* < 0.01.

obese women compared to the control women (Fig. 3B: *p* < 0.05). Also different to the decidua parietalis no differences in M1 macrophage levels (HLA-DR⁺CD163⁻) were found in the decidua basalis between obese women and control women (Fig. 3, Table 3). Similar to the

decidua parietalis, the geo MFI of the M1 marker HLA-DR had a trend (*p* = 0.09) to be lower in the obese women compared to the control women (Fig. 3F, Table 3). Similar to the decidua parietalis no differences were found for levels of M2 (HLA-DR⁻CD163⁺) macrophages

Table 3
Average percentages of macrophages.

	Decidua parietalis		Decidua basalis	
	Control group BMI < 25 (n = 10)	Obese group BMI > 30 (n = 10)	Control group BMI < 25 (n = 10)	Obese group BMI > 30 (n = 10)
Total macrophages/leukocytes	47.3 ± 24.3	51.9 ± 26.2	33.3 ± 13.0*	26.3 ± 10.5*
M1/total macrophages	5.3 ± 4.4*	4.3 ± 0.8*	15.6 ± 10.3	15.7 ± 9.3
M2/total macrophages	5.5 ± 2.3	5.5 ± 8.7	1.7 ± 1.9	2.0 ± 1.4
Decidual macrophages/total macrophages	84.22 ± 19.0	82.21 ± 19.2	8.3 ± 11.7	11.7 ± 13.2
M1/decidual macrophages	5.2 ± 2.8**	4.3 ± 1.4**	12.8 ± 5.0	14.1 ± 9.1
M2/decidual macrophages	6.1 ± 4.2	5.0 ± 3.8	10.1 ± 4.8	9.4 ± 4.6
Non-decidual macrophages	15.8 ± 19.1	17.8 ± 19.2	91.7 ± 11.7	88.3 ± 13.2
M1/non-decidual macrophages	6.3 ± 7.1	4.6 ± 4.1	14.5 ± 11.6	16.0 ± 8.7
M2/non-decidual macrophages	9.1 ± 10.7	12.9 ± 11.6	1.1 ± 1.2	1.0 ± 1.5

M1 is defined as HLA-DR⁺CD163⁻ and M2 is defined as HLA-DR⁻CD163⁺. Data were described as median ± interquartile range. * *p* < 0.05, ** *p* < 0.01 compared to control using Mann-Whitney U test.

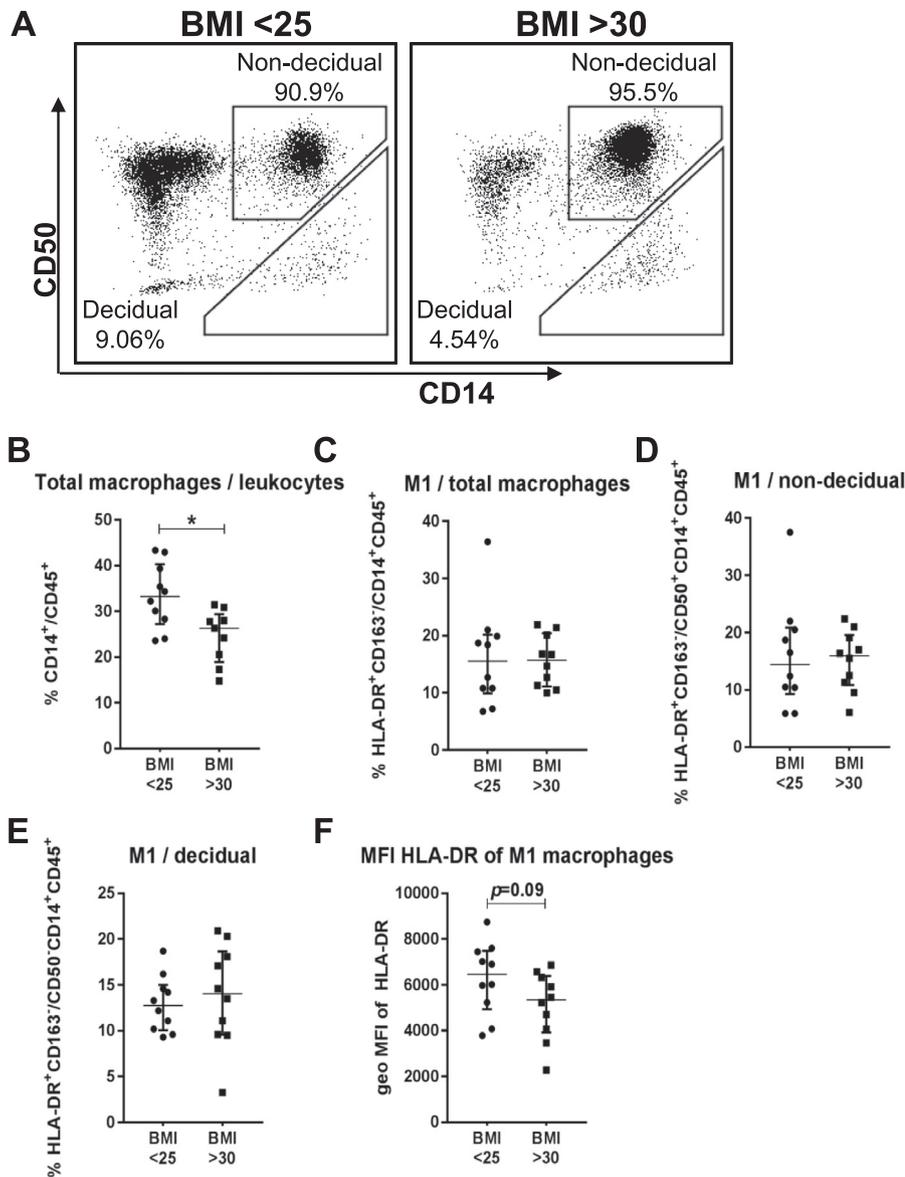


Fig. 3. Macrophages in decidua basalis of women with a BMI < 25 kg/m² or with a BMI > 30 kg/m². Gating of decidual macrophages (CD50⁻CD14⁺) and non-decidual macrophages (CD50⁺CD14⁺) in different groups (A). Proportions of total macrophages (CD14⁺) of leukocytes (CD45⁺) (B), proportions of M1 macrophages (HLA-DR⁺CD163⁻) of total macrophages (C), proportions of M1 macrophages of non-decidual macrophages (CD50⁺CD14⁺CD45⁺) (D), and proportions of M1 macrophages of decidual macrophages (CD50⁻CD14⁺CD45⁺) (E). Geometric mean fluorescence intensity (geo MFI) of M1 macrophages of total macrophages (F). Symbols represent values of individual decidua. Horizontal lines are medians ± interquartile range. Analysis by Mann-Whitney *U* test; * = *p* < 0.05.

between obese and control women (Table 3).

3.4. Higher levels of macrophages and macrophage subsets in the decidua parietalis compared to the decidua basalis

To analyse the different macrophage mediated immune response in the decidua basalis and parietalis we investigated whether levels of macrophages and macrophage subsets differed between the decidua parietalis and decidua basalis (Fig. 4, Table 3). The proportion of CD14⁺ macrophages in leukocytes (CD45⁺) was nearly twice as high in the decidua parietalis than in the decidua basalis (*p* < 0.001; Fig. 4B). Within the total macrophage population (CD14⁺CD45⁺), the decidual macrophage fraction (CD50⁻CD14⁺CD45⁺) was higher in the decidua parietalis than decidua basalis (*p* < 0.001; Fig. 4C). M1 (HLA-DR⁺CD163⁻) and M2 (HLA-DR⁻CD163⁺) macrophage levels differed between the decidua parietalis and basalis with lower levels of M1 (HLA-DR⁺CD163⁻) macrophages in the decidua parietalis compared to the basalis (*p* < 0.001; Fig. 4CFI), and higher levels of M2 macrophages in the decidua parietalis compared to the basalis (*p* < 0.001; Fig. 4DGJ).

4. Discussion

In this study we showed that obese women with an uncomplicated term pregnancy have lower levels of M1 (HLA-DR⁺CD163⁻) macrophages, considered to be pro-inflammatory, in the decidua parietalis compared to the decidua parietalis of control women. These lower levels of M1 (HLA-DR⁺CD163⁻) macrophages were accompanied by a trend towards a lower geometric mean fluorescence intensity (geo MFI) of the M1 marker HLA-DR. We also showed lower proportions of M1 (HLA-DR⁺CD163⁻) macrophages, and higher proportions of M2 (HLA-DR⁻CD163⁺) macrophages in the decidua parietalis compared to the decidua basalis.

Earlier studies have found higher levels of CD80⁺ macrophages in obese adipose tissue [40–42], and higher levels of pro-inflammatory cytokines IL-1, TNF-α and IL-6 in placental tissue of obese women have been found [3–5,41]. Our findings of lower levels of M1 (HLA-DR⁺CD163⁻) macrophages in the decidua parietalis of obese pregnant women could be explained by several factors. Firstly, we did only include uncomplicated term pregnancies. The lower levels of M1 (HLA-DR⁺CD163⁻) macrophages might therefore indicate a successful compensatory mechanism for the increased inflammatory state in obese

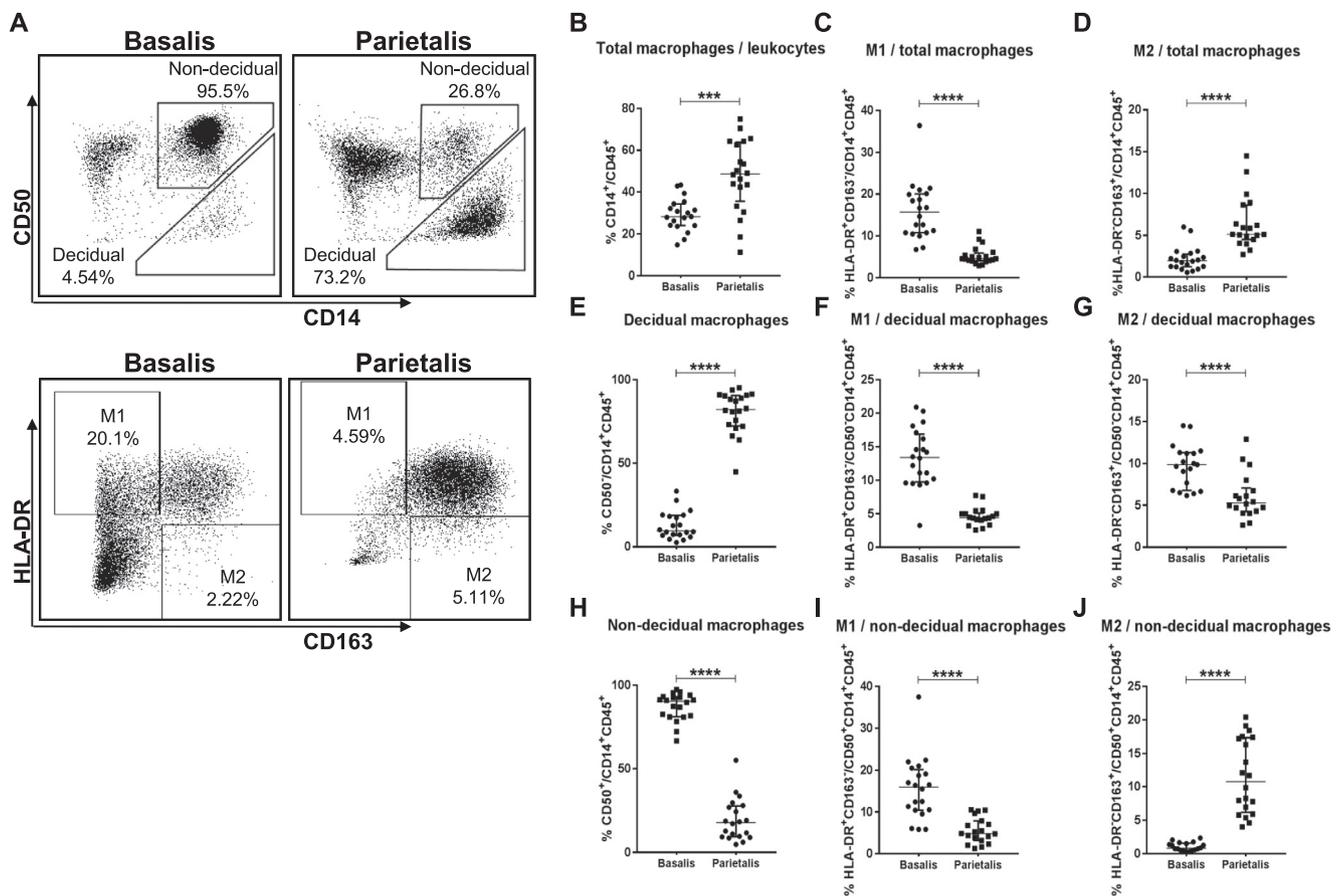


Fig. 4. Macrophages in decidua parietalis and decidua basalis. Gating of decidual macrophages (CD50⁻CD14⁺CD45⁺), non-decidual macrophages (CD50⁺CD14⁺CD45⁺), M1 macrophages (HLA-DR⁺CD163⁻) and M2 macrophages (HLA-DR⁻CD163⁺) in both decidual tissues (A). Proportions of macrophages (CD14⁺) of total leukocytes (CD45⁺) (B), M1 macrophages of total macrophages (C), M2 macrophages of total macrophages (CD14⁺CD45⁺) (D). Proportions of decidual macrophages of total macrophages (E), M1 and M2 macrophages of decidual macrophages (F-G). Proportions of non-decidual macrophages of total macrophages (H), M1 and M2 macrophages of non-decidual macrophages (I-J). Horizontal lines are medians \pm interquartile range. Analysis by Mann-Whitney *U* test; *** = $p < 0.001$, **** = $p < 0.0001$.

individuals. It could be that in pregnancies in obese women in whom this compensation mechanism fails, pregnancy will have a complicated outcome. Secondly, our macrophage gating and classification strategy differs from earlier studies. Based on recent studies we classified M1 and M2 macrophages based on HLA-DR or CD163 expression [16,39]. A potential limitation of macrophage studies in general is the sometimes troublesome characterization of macrophages in different subsets by phenotypic markers. The gating strategy used in this study ensures two different macrophage subsets without the existence of double cell populations. We found the lower levels of M1 (HLA-DR⁺CD163⁻) macrophages in obese women both in the total macrophage population (CD14⁺CD45⁺) and in the decidual macrophage population (CD50⁻CD14⁺CD45⁺) in the decidua parietalis. These findings were further strengthened with a trend towards lower geo MFI ($p = 0.06$) of the M1 marker HLA-DR in obese women in both decidual tissues.

Previous studies have indicated an inflammatory environment occurring in obesity, such as higher levels of pro-inflammatory cytokines IL-1, TNF- α and IL-6 in placental tissue of obese women [3–5,41], this could indicate the need for compensatory mechanisms to ensure successful pregnancy outcome. In obesity, adipose tissue macrophages (ATM) are thought to be important in immune mediated obesity complications [43]. These ATMs are known to differ from other tissue resident macrophages, including (phenotypic) characterization [43]. The role and function of these ATMs in pregnancy, and if these ATMs are recruited into the placenta during pregnancy in obese women is, to our knowledge, not known.

Earlier studies have also found differences between immune cell levels in the decidua basalis and parietalis in pregnancy complications [16,27,44]. A physiologic reason for differences in immune subsets, and with that for the lower levels of M1 (HLA-DR⁺CD163⁻) macrophages in the decidua parietalis we found, and the lack of macrophage differences in the basalis needs to be established. A role for decidual stromal cells can be expected since these cells form the structures of both decidua parietalis and decidua basalis [44]. It is also possible that the two layers differ in homing and recruitment of macrophages which is mediated by adhesion molecules, expression of molecules on trophoblast (such as HLA), and macrophage expression patterns itself. Future studies are necessary to determine the mechanisms involved in the regional trafficking of macrophages in the term decidua [45,46]. Solders et al. have hypothesized that a possible physiologic explanation for differences in the decidua basalis and parietalis is the increased vascularization in the decidua basalis [44]. This causes recruitment of systemic immune cells and provides immunologic protection against blood-related pathogens. The decidua parietalis has not such an invasive vascularization and rather contains stationary distributed immune cells, thought to be of importance in the prevention of mucosal infectious agents [44]. This could result in a higher vulnerability of macrophages in the decidua parietalis compared to decidua basalis towards factors associated with obesity. The current study does indicate different macrophage subsets in the decidua basalis and parietalis, as the decidual macrophage fraction (CD50⁻CD14⁺CD45⁺) of the total macrophages is significantly higher (> 10-fold) in the decidua

parietalis compared to the decidua basalis. Previous studies have shown that especially the CD50 negative macrophage fraction can be considered as decidual macrophages CD50 [24,25]. The exact physiologic involvement for the differences found in macrophage populations needs to be elucidated in further studies.

We identified differences in all investigated macrophage subsets in the decidua parietalis as compared to the decidua basalis. These findings suggest regional influences of trophoblasts on decidual cells. It also supports the importance of investigating the two decidual layers separately which is not performed in many studies [12,38,44,45]. Regulatory T cells (Tregs) have been demonstrated to be increased in the decidua parietalis compared to decidua basalis [38,44]. Tregs induce M2 macrophages by immune response inhibition via e.g. TGF- β and IL-10 and this could cause the rise in active M2 macrophages in the decidua parietalis [20,47]. As described above it has been hypothesized that a possible physiologic explanation for differences in the decidua basalis and parietalis is the increased vascularization in the basalis [44].

The (spontaneous) onset of labor might also be a variable of importance in the explanation of the found differences between the two layers. In our study there was no difference in the percentage of labor induction between the control and obese pregnant group. Recent studies show recruitment of pro-inflammatory macrophages to the cervix and decidua in the peripartum period and decreased expression of the chemokine CCL-18, known to induce M2 macrophages [16,25,26,30,48]. Labor might induce morphological influx of specific immune cells to the decidua parietalis, possibly related to the initiation of labor [44,49]. This phenomenon is however not yet established for the complete macrophage spectrum. A deeper understanding of the immunological involvement of macrophage subsets in the decidua parietalis and basalis might allow the development of strategies to improve pregnancy outcome and more specific influence trophoblastic immune regulation.

A potential limitation of macrophage studies is the sometimes troublesome characterization of macrophages in different subsets. The classic division in pro-inflammatory M1 and anti-inflammatory M2 macrophages is not as strict as suggested, and overlapping phenotypes exist [29]. In mice adipose tissue, both M1 and M2 macrophage transcriptional genes, associated with up regulation of both M1 and M2 profiles, are observed [50]. This suggests that the classic two subsets symbolize the extremes of one cell population. In macrophages isolated from first trimester decidual tissue, it is shown that macrophages are not simply attributable to one subgroup or another [48]. In our population high levels of macrophages positive for both markers were found (HLA-DR⁺CD163⁺), this population was especially prominent in the decidua parietalis, in which the macrophage population (CD14⁺CD45⁺) consisted of the highest decidual macrophage fraction (CD50⁻CD14⁺CD45⁺). Our results therefore confirm previous studies in which it was shown that decidual macrophages are not simply attributable to one subgroup of macrophages or another. A more precise definition of macrophage subtypes by appropriate combinations of antibodies and functional studies needs validation in future studies.

In this study only term placentas of uncomplicated pregnancies were included. Therefore, the lower levels of M1 (HLA-DR⁺CD163⁻) macrophages we found in the decidua parietalis of obese women could be a compensatory mechanism towards the pro-inflammatory state associated with obesity. As obesity is associated with adverse pregnancy outcomes, it could be that if this compensatory mechanism fails, an adverse pregnancy outcome is imminent. Future studies analyzing the mechanisms which determine if a pregnancy in an obese woman has a healthy or complicated pregnancy outcome are needed.

5. Conclusion

In conclusion, maternal obesity is associated with lower levels of M1 (HLA⁻DR⁺CD163⁻) macrophages in the decidua parietalis. Further

studies have to elucidate if these lower levels of M1 macrophages in obese women are a compensatory mechanism to compensate for increased pro-inflammatory state in obesity. Since the decidua basalis and decidua parietalis show different immunologic regulations of the macrophage populations, this study indicates the importance of investigating decidual layers separately.

Conflict of interest

The authors have no conflicts of interests to declare.

Funding sources

This work was supported by a Mandema Stipendium awarded to J.R. Prins.

References

- [1] N.M. Wagner, G. Brandhorst, F. Czepluch, M. Lankeit, C. Eberle, S. Herzberg, V. Faustin, J. Riggert, M. Oellerich, G. Hasenfuss, S. Konstantinides, K. Schäfer, Circulating regulatory T cells are reduced in obesity and may identify subjects at increased metabolic and cardiovascular risk, *Obesity* 21 (2013) 461–468, <https://doi.org/10.1002/oby.20087>.
- [2] R. Travers, A. Motta, J. Betts, A. Bouloumié, D. Thompson, The impact of adiposity on adipose tissue-resident lymphocyte activation in humans, *Int. J. Obes.* 39 (2014) 762–769, <https://doi.org/10.1038/ijo.2014.195>.
- [3] J.C. Challier, S. Basu, T. Bintein, J. Minium, K. Hotmire, P.M. Catalano, S. Hauguel-de Mouzon, Obesity in pregnancy stimulates macrophage accumulation and inflammation in the placenta, *Placenta* 29 (2008) 274–281, <https://doi.org/10.1016/j.placenta.2007.12.010>.
- [4] R.M.I. Wilson, The impact of maternal obesity during pregnancy on offspring immunity, *Mol. Cell. Endocrinol.* 8 (2016) 134–142, <https://doi.org/10.1002/aur.1474.Replication>.
- [5] F.T. Spradley, A.C. Palei, J.P. Granger, Immune mechanisms linking obesity and preeclampsia, *Biomolecules* 5 (2015) 3142–3176, <https://doi.org/10.3390/biom5043142>.
- [6] L. Myatt, A. Maloyan, Obesity and placental function, *Semin. Reprod. Med.* 34 (2016) 42–49, <https://doi.org/10.1055/s-0035-1570027>.
- [7] K.A. Roberts, S.C. Riley, R.M. Reynolds, S. Barr, M. Evans, A. Statham, K. Hor, H.N. Jabbour, J.E. Norman, F.C. Denison, Placental structure and inflammation in pregnancies associated with obesity, *Placenta* 32 (2011) 247–254, <https://doi.org/10.1016/j.placenta.2010.12.023>.
- [8] S. Basu, M. Haghiaç, P. Surace, J.-C. Challier, M. Guerre-Millo, K. Singh, T. Waters, J. Minium, L. Presley, P.M. Catalano, S. Hauguel-de Mouzon, Pregravid obesity associates with increased maternal endotoxemia and metabolic inflammation, *Obesity* 19 (2011) 476–482, <https://doi.org/10.1038/oby.2010.215>.
- [9] L. Poston, R. Caleyachetty, S. Cnattingius, C. Corvalán, R. Uauy, S. Herring, M.W. Gillman, Preconceptional and maternal obesity: epidemiology and health consequences, *Lancet Diab. Endocrinol.* 4 (2016) 1025–1036, [https://doi.org/10.1016/S2213-8587\(16\)30217-0](https://doi.org/10.1016/S2213-8587(16)30217-0).
- [10] S.S. Pereira, J.I. Alvarez-Leite, Low-grade inflammation, obesity, and diabetes, *Curr. Obes. Rep.* 3 (2014) 422–431, <https://doi.org/10.1007/s13679-014-0124-9>.
- [11] M.M. Faas, P. de Vos, Uterine NK cells and macrophages in pregnancy, *Placenta* 56 (2017) 44–52, <https://doi.org/10.1016/j.placenta.2017.03.001>.
- [12] U. Repnik, T. Tilburgs, D.L. Roelen, B.J. van der Mast, H.H.H. Kanhai, S. Scherjon, F.H.J. Claas, Comparison of macrophage phenotype between decidua basalis and decidua parietalis by flow cytometry, *Placenta* 29 (2008) 405–412, <https://doi.org/10.1016/j.placenta.2008.02.004>.
- [13] D. Schonkeren, G. Swings, D. Roberts, F. Claas, E. de Heer, S. Scherjon, Pregnancy close to the edge: an immunosuppressive infiltrate in the chorionic plate of placentas from uncomplicated egg cell donation, *PLoS One* 7 (2012) 1–8, <https://doi.org/10.1371/journal.pone.0032347>.
- [14] R.H. McIntire, M.G. Petroff, T.A. Phillips, J.S. Hunt, In vitro models for studying human uterine and placental macrophages, *Meth. Mol. Med.* 122 (2006) 123–148.
- [15] M. Weiss, K. Blazek, A.J. Byrne, D.P. Perocheau, I.A. Udalova, IRF5 is a specific marker of inflammatory macrophages in vivo, *Mediat. Inflamm.* 2013 (2013), <https://doi.org/10.1155/2013/245804>.
- [16] Y. Xu, R. Romero, D. Miller, L. Kadam, T.N. Mial, O. Plazyo, V. Garcia-Flores, S.S. Hassan, Z. Xu, A.L. Tarca, S. Drewlo, N. Gomez-Lopez, An M1-like macrophage polarization in decidual tissue during spontaneous preterm labor that is attenuated by rosiglitazone treatment, *J. Immunol.* 196 (2016) 2476–2491, <https://doi.org/10.4049/jimmunol.1502055>.
- [17] S. Hauguel-de Mouzon, M. Guerre-Millo, The placenta cytokine network and inflammatory signals, *Placenta* 27 (2006) 794–798, <https://doi.org/10.1016/j.placenta.2005.08.009>.
- [18] M.X. Tang, X.H. Hu, Z.Z. Liu, J. Kwak-Kim, A.H. Liao, What are the roles of macrophages and monocytes in human pregnancy? *J. Reprod. Immunol.* 112 (2015) 73–80, <https://doi.org/10.1016/j.jri.2015.08.001>.
- [19] T. Krausgruber, K. Blazek, T. Smallie, N. Alzabin, H. Lockstone, N. Sahgal, T. Hussell, M. Feldmann, I.A. Udalova, IRF5 promotes inflammatory macrophage

- polarization and TH1-TH17 responses, *Nat. Immunol.* 12 (2011) 231–238, <https://doi.org/10.1038/ni.1990>.
- [20] G. Liu, H. Ma, L. Qiu, L. Li, Y. Cao, J. Ma, Y. Zhao, Phenotypic and functional switch of macrophages induced by regulatory CD4+CD25+ T cells in mice, *Immunol. Cell Biol.* 89 (2011) 130–142, <https://doi.org/10.1038/icb.2010.70>.
- [21] S. Goerdts, C.E. Orfanos, Other functions, other genes: alternative activation of antigen-presenting cells, *Immunology* 10 (1999) 137–142 S1074-7613(00)80014-X [pii].
- [22] R.M. Anthony, J.F. Urban, F. Alem, H.A. Hamed, C.T. Rozo, J.L. Boucher, N. Van Rooijen, W.C. Gause, Memory TH2 cells induce alternatively activated macrophages to mediate protection against nematode parasites, *Nat. Med.* 12 (2006) 955–960, <https://doi.org/10.1038/nm1451>.
- [23] S. Gordon, Alternative activation of macrophages, *Nat. Rev. Immunol.* 3 (2003) 23–35, <https://doi.org/10.1038/nri978>.
- [24] J. Svensson, M.C. Jenmalm, A. Matussek, R. Geffers, G. Berg, J. Ernerudh, Macrophages at the fetal-maternal interface express markers of alternative activation and are induced by M-CSF and IL-10, *J. Immunol.* (2011), <https://doi.org/10.4049/jimmunol.1100130>.
- [25] C. Gustafsson, J. Mjösberg, A. Matussek, R. Geffers, L. Matthiesen, G. Berg, S. Sharma, J. Buer, J. Ernerudh, Gene expression profiling of human decidual macrophages: evidence for immunosuppressive phenotype, *PLoS One* 3 (2008) 1–9, <https://doi.org/10.1371/journal.pone.0002078>.
- [26] T. Nagamatsu, D.J. Schust, The contribution of macrophages to normal and pathological pregnancies, *Am. J. Reprod. Immunol.* 63 (2010) 460–471, <https://doi.org/10.1111/j.1600-0897.2010.00813.x>.
- [27] D. Schonkeren, M.L. Van Der Hoorn, P. Khedoe, G. Swings, E. Van Beelen, F. Claas, C. Van Kooten, E. De Heer, S. Scherjon, Differential distribution and phenotype of decidual macrophages in preeclamptic versus control pregnancies, *Am. J. Pathol.* 178 (2011) 709–717, <https://doi.org/10.1016/j.ajpath.2010.10.011>.
- [28] H. Wang, M. He, Y. Hou, S. Chen, X. Zhang, M. Zhang, X. Ji, Role of decidual CD14+ macrophages in the homeostasis of maternal-fetal interface and the differentiation capacity of the cells during pregnancy and parturition, *Placenta* 38 (2016) 76–83, <https://doi.org/10.1016/j.placenta.2015.12.001>.
- [29] E. Muraille, O. Leo, M. Moser, Th1/Th2 paradigm extended: macrophage polarization as an unappreciated pathogen-driven escape mechanism? *Front. Immunol.* 5 (2014) 1–12, <https://doi.org/10.3389/fimmu.2014.00603>.
- [30] M.B. Brown, M. von Chamier, A.B. Allam, L. Reyes, M1/M2 macrophage polarity in normal and complicated pregnancy, *Front. Immunol.* 5 (2014) 1–10, <https://doi.org/10.3389/fimmu.2014.00606>.
- [31] S. Hamilton, Y. Oomomian, G. Stephen, O. Shynlova, C.L. Tower, A. Garrod, S.J. Lye, R.L. Jones, Macrophages infiltrate the human and rat decidua during term and preterm labor: evidence that decidual inflammation precedes labor, *Biol. Reprod.* 86 (2012) 1–9, <https://doi.org/10.1095/biolreprod.111.095505>.
- [32] A.E. Frias, T.K. Morgan, A.E. Evans, J. Rasanen, K.Y. Oh, K.L. Thornburg, K.L. Grove, Maternal high-fat diet disturbs uteroplacental hemodynamics and increases the frequency of stillbirth in a nonhuman primate model of excess nutrition, *Endocrinology* 152 (2011) 2456–2464, <https://doi.org/10.1210/en.2010-1332>.
- [33] M.J. Zhu, M. Du, P.W. Nathanielsz, S.P. Ford, Maternal obesity up-regulates inflammatory signaling pathways and enhances cytokine expression in the mid-gestation sheep placenta, *Placenta* 31 (2010) 387–391, <https://doi.org/10.1016/j.placenta.2010.02.002>.
- [34] S. Basu, S. Hauguel-de Mouzon, P. Leahy, J. Minium, P. Catalano, J.-C. Challier, Placenta macrophages of obese women originate from infiltrating maternal monocytes, *Am. J. Obstet. Gynecol.* 204 (2011) S13–S14, <https://doi.org/10.1016/j.ajog.2010.10.031>.
- [35] S.M. Leon-Garcia, H.A. Roeder, K.K. Nelson, X. Liao, D.P. Pizzo, L.C. Laurent, M.M. Parast, D.Y. LaCoursiere, Maternal obesity and sex-specific differences in placental pathology, *Placenta* (2016), <https://doi.org/10.1016/j.placenta.2015.12.006>.
- [36] A. Van Egmond, C. Van der Keur, G.M.J.S. Swings, E. Van Beelen, L. Van Zijl, S.A. Scherjon, F.H.J. Claas, Preservation of human placenta facilitates multicenter studies on the local immune response in normal and aberrant pregnancies, *J. Reprod. Immunol.* 98 (2013) 29–38, <https://doi.org/10.1016/j.jri.2013.03.001>.
- [37] Committee for Guidelines in Research (COREON), Human Tissue and Medical Research: Code of Conduct for responsible use, 2011.
- [38] T. Tilburgs, D.L. Roelen, B.J. van der Mast, J.J. van Schip, C. Kleijburg, G.M. de Groot-Swings, H.H.H. Kanhai, F.H.J. Claas, S.A. Scherjon, Differential distribution of CD4+CD25bright and CD8+CD28– T-cells in decidua and maternal blood during human pregnancy, *Placenta* 27 (2016) 47–53, <https://doi.org/10.1016/j.placenta.2005.11.008>.
- [39] S. Shimada, Y. Ebina, N. Iijima, M. Deguchi, H. Yamada, Decidual CD68+HLA-DR+CD133–M1 macrophages increase in miscarriages with normal fetal chromosome, *Am. J. Reprod. Immunol.* e12791 (2017), <https://doi.org/10.1111/aji.12791>.
- [40] F.C. Denison, K.A. Roberts, S.M. Barr, J.E. Norman, Obesity, pregnancy, inflammation, and vascular function, *Reproduction* 140 (2010) 373–385, <https://doi.org/10.1530/REP-10-0074>.
- [41] L. Boutens, R. Stienstra, Adipose tissue macrophages: going off track during obesity, *Diabetologia* 59 (2016) 879–894, <https://doi.org/10.1007/s00125-016-3904-9>.
- [42] S. Basu, M. Haghiaç, P. Surace, J.-C. Challier, M. Guerre-Millo, K. Singh, T. Waters, J. Minium, L. Presley, P.M. Catalano, S. Hauguel-de Mouzon, Pregravid obesity associates with increased maternal endotoxemia and metabolic inflammation, *Obesity* (Silver Spring) 19 (2011) 476–482, <https://doi.org/10.1038/oby.2010.215>.
- [43] L. Russo, C.N. Lumeng, C. Lumeng, Title Properties and functions of adipose tissue macrophages in obesity, 1 (n.d.) 0–3. doi:10.1111/imm.13002.
- [44] M. Solders, L. Gorchs, S. Gidlöf, E. Tiblad, A. Lundell, H. Kaipe, Maternal adaptive immune cells in decidua parietalis display a more activated and coinhibitory phenotype compared to decidua basalis, *Stem Cells Int.* 2017 (2017).
- [45] A. Sindram-Trujillo, S. Scherjon, H. Kanhai, D. Roelen, F. Claas, Increased T-cell activation in decidua parietalis compared to decidua basalis in uncomplicated human term pregnancy, *Am. J. Reprod. Immunol.* 49 (2003) 261–268, <https://doi.org/10.1034/j.1600-0897.2003.00041.x>.
- [46] A.P. Sindram-Trujillo, S.A. Scherjon, P.P. Van Hulst-van Miert, J.J. Van Schip, H.H.H. Kanhai, D.L. Roelen, F.H.J. Claas, Differential distribution of NK cells in decidua basalis compared with decidua parietalis after uncomplicated human term pregnancy, *Hum. Immunol.* 64 (2003) 921–929, [https://doi.org/10.1016/S0198-8859\(03\)00170-8](https://doi.org/10.1016/S0198-8859(03)00170-8).
- [47] A. Schmidt, X.-M. Zhang, R.N. Joshi, S. Iqbal, C. Wahlund, S. Gabrielson, R.A. Harris, J. Tegnér, Human macrophages induce CD4+Foxp3+ regulatory T cells via binding and re-release of TGF-β, *Immunol. Cell Biol.* 94 (2016) 747–762, <https://doi.org/10.1038/icb.2016.34>.
- [48] A. Erlebacher, Immunology of the Maternal-Fetal Interface, *Annu. Rev. Immunol.* 31 (2013) 387–411, <https://doi.org/10.1146/annurev-immunol-032712-100003>.
- [49] L. Marcellin, T. Schmitz, M. Messaoudene, D. Chader, C. Parizot, S. Jacques, J. Delaire, J. Gogusev, A. Schmitt, C. Lesaffre, M. Breuille-Fouché, A. Caignard, D. Vaiman, F. Goffinet, D. Cabrol, G. Gorochov, C. Méhats, Immune modifications in fetal membranes overlying the cervix precede parturition in humans, *J. Immunol.* 198 (2017) 1345–1356, <https://doi.org/10.4049/jimmunol.1601482>.
- [50] M.E. Shaul, G. Bennett, K.J. Strissel, A.S. Greenberg, M.S. Obin, Adipose tissue macrophages during high-fat diet – induced obesity in mice, *Diabetes* 59 (2010) 1171–1181, <https://doi.org/10.2337/db09-1402>.