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## Immunological challenges during pregnancy : preeclampsia and egg donation

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Differential distribution and phenotype of decidual macrophages in preeclamptic versus control pregnancies



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## Abstract

**Background:** Tolerance towards the semi-allogeneic fetus is a complex and basically unrevealed phenomenon. As macrophages are an abundant cell population in the human decidua, changes in distribution or phenotype may be involved in the development of preeclampsia. The aim of this study was to assess the distribution and phenotype of macrophages in preterm preeclamptic, preterm control, and term control placentas.

**Methods:** Placentas of preterm preeclamptic (n=6), of preterm control (n=5), and of term control pregnancies (n=6) were sequentially immunohistochemically stained for CD14, CD163, DC SIGN and IL-10. The distributions of CD14+, CD163+, DC SIGN+, IL-10+, CD163+/CD14+, DC SIGN+/CD14+ and Flt-1/CD14+ cells were determined by double staining and by digital image analysis of sequential photomicrographs.

**Results:** CD14 and CD163 expression was significantly increased in preterm preeclamptic decidua basalis compared with preterm control pregnancies (p=0.0006 and p=0.034 respectively). IL-10 expression was significantly lower in the decidua parietalis of preterm preeclamptic pregnancies compared with preterm control pregnancies (p=0.03). The ratio CD163/CD14 was significantly lower in the decidua basalis (p=0.0293) and the ratio of DC SIGN/CD14 was significantly higher decidua basalis (p=<0.0001) and parietalis (p=<0.0001) of preterm preeclamptic compared with preterm control pregnancies. CD14+ macrophages did express Flt-1.

**Conclusion:** Alterations in distribution and phenotype of macrophages in the decidua of preterm preeclamptic pregnancies compared to control pregnancies may contribute to the pathogenesis of preeclampsia.

## Introduction

Maternal immune tolerance towards the semi-allogeneic fetus and placenta is important in uncomplicated human pregnancy. Maternal immune cells at the fetomaternal interface are directly exposed to fetal antigens at three locations [1]. First, the maternal tissue lining the fetal membranes, the decidua parietalis, interact with the trophoblast cells of the chorion. Second, the maternal part of the placenta, the decidua basalis, is infiltrated by invading extravillous trophoblast. Third, after the establishment of the uteroplacental circulation, maternal peripheral blood contacts with syncytiotrophoblast. Several mechanisms, some of them implying a special role for macrophages at the three interfaces, have been postulated to promote an immunomodulatory state [2,3].

Macrophages are antigen-presenting cells which account for the second most numerous type of leukocytes in the human decidua [4]. They are mononuclear phagocytotic cells involved in the innate and adaptive immune system. Macrophages promote inflammation by production of inflammatory molecules during an innate immune response and, are able to present antigens to T cells as part of the adaptive immune system. Macrophages may have a role in immunosuppression in the human decidua, as suggested by their ability to suppress a one-way mixed lymphocyte reaction [5]. Furthermore, macrophages express costimulation molecules CD80 and CD86 in low levels and they express indoleamine 2,3-dioxygenase, both preventing T lymphocyte activation [6]. An alteration in the quantity or distribution of these cells may be involved in the development of preeclampsia. Preeclampsia is a relatively common but potentially dangerous disorder in human pregnancy, leading to maternal and neonatal morbidity and mortality. It affects 1-7% of nulliparous women who have a three times higher risk than multiparous women [7,8]. The disease is characterized by inadequate transformation of the spiral arteries [9] and generalized maternal sFlt-1-mediated endothelial cell dysfunction [10]. Furthermore, immunologic factors are involved in the pathogenesis of preeclampsia since earlier exposure with paternal antigens decreases the risk of preeclampsia [11,12].

The exact role of macrophages in the human decidua and their function in preeclampsia remains unknown. The numbers of macrophages have been studied by several groups with varying results. A reduction in the number of CD14+ macrophages [13], no alteration [14] and increased numbers of macrophages [15] have been found in decidua from preeclampsia compared to control women. Because of these discrepancies in the literature we intended to study the role and distribution of macrophages in control and preeclamptic decidua. For phenotypic characterization of the macrophage subsets three different markers were tested. CD14, a glycosylphosphatidylinositol-anchored membrane protein, is present on monocytes and macrophages. The macrophage scavenging receptor, CD163 is a mononuclear phagocyte restricted cell surface glycoprotein antigen present on type 2 macrophages (M2 cells) which have been reported to exert an anti-inflammatory function [16]. Gene expression profiling shows that human decidua mainly contains M2 cells, which contribute to the immunosuppressive state favorable to the maintenance of the semi-allogeneic fetus [17]. In contrast to M2 cells, macrophages stimulated with Th1 cytokines polarize toward a pro-inflammatory type 1 macrophages (M1 cells). These cells are able to defend upon uteroplacental infections but do not contribute to the tolerance of the fetus [18]. Furthermore, we used the dendritic cell-specific marker ICAM3-grabbing nonintegrin (DC SIGN) for phenotypic characterization. DC SIGN is highly expressed on immature DCs but also present on macrophages in the human decidua [19,20].

In addition we stained the IL-10 and Flt-1 expression by immunohistochemistry in the decidua basalis and parietalis. IL-10 is an immunosuppressive molecule, produced by T cells, macrophages/monocytes and B cells. This cytokine is spontaneously produced in high levels by decidual macrophages [6]. It is a Th2 type cytokine and appears to be pregnancy protective [21]. Decreased villous trophoblast staining of IL-10 has been demonstrated in women with

preeclampsia compared to normal pregnancy with correlated gestational age [22].

Coexpression of CD14 and CD68 as a general macrophage marker, with either CD163, DC SIGN or sFlt was studied to define the phenotype of cells. We determined the number and type of macrophages in decidua of preterm preeclamptic, preterm control, and term control pregnancies and defined the natural polarization of decidual macrophages and alterations of the phenotype of these cells.

## Material and Methods

### Patient selection

After a pilot study of five preterm preeclamptic and five term control placentas, six preterm preeclamptic, five preterm control and six term control placentas were collected. Criteria for inclusion in the preeclamptic group were presence of hypertension (diastolic blood pressure  $\geq$  95 mm Hg), proteinuria ( $>$  0.3 gr/l/24 hours) and a gestational age below 34 0/7 weeks. Term placentas were collected from healthy women after normal, uncomplicated pregnancies of 37-42 weeks gestational age. Preterm placentas were collected if delivered before 34 weeks gestational age after an uncomplicated pregnancy without any signs of infection. This group contained a quadruplet of which the placentas were analyzed as separate. The values obtained in the singleton preterm control placenta were in the same range as those observed in the placentas of the quadruplet pregnancy. No significant differences were present between the singleton preterm control placenta and the quadruplet preterm control placentas for the stainings of CD14, CD163 and DC SIGN as well as in the decidua basalis or parietalis (data not shown). Tissue samples were collected within five hours after the time of delivery of the placenta after primary caesarean section or vaginal delivery. The study was approved by the ethics committee of the Leiden University Medical Center (LUMC) and informed consent of every patient was obtained.

### Immunohistochemistry

Tissue blocks of the placenta and rolls of fetal membranes were taken at three locations, fixed in 4% formalin and routinely embedded in paraffin. Sequential serial sections (4 $\mu$ m-thick) were cut on adhesive coated glasses and dried overnight at 37°C. Tissue sections were deparaffinized and hydrated by xylene in decreasing alcohol concentration to demi-H<sub>2</sub>O. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 20 minutes. After a wash step with demi-H<sub>2</sub>O, antigen retrieval was performed by boiling the sections for 10 minutes in citrate buffer (pH 6.0). The slides were cooled down for 20 minutes followed by another wash step. The optimal dilution for each primary antibody was determined in positive decidual tissue selected on the basis of maximal specific reactivity and minimal background staining (Table 1). As a control the primary antibody was replaced by normal serum. The primary antibody was incubated for one hour at room temperature at the appropriate dilutions in PBS with 1% BSA (except for IL-10, which was pre treated with normal goat serum for 30 minutes and incubated overnight). After washing three times in PBS the slides were incubated 30 minutes with Envision (DAKO, North America Inc, USA). Another wash step was followed by 5 minutes incubation with diaminobenzidine (DAB, DAKO Cytomation). Demi-H<sub>2</sub>O was used to stop the reaction. The tissue sections were subsequently counterstained with haematoxylin (SIGMA, Switzerland, Steinheim). The slides were mounted in mounting medium (Surgipath Medical Ind., Inc. Richmond) and covered.

Antibody	Isotype	Dilution	Source
CD14	IgG2a	1:200	Novocastra, Newcastle, United Kingdom
CD68	IgG1	1:250	DAKO, North America Inc, USA
CD163	IgG1	1:20	Abcam, Cambridge, United Kingdom
DC SIGN (CD209)	IgG2b	1:4000	Miltenyi Biotecs MACS, Bergisch Gladbach, Germany
IL-10	Polyclonal IgG	1:50	Hycult Biotech, Uden, The Netherlands
Flt-1	Polyclonal IgG	1:250	Santa cruz, Biotechnology Inc, Heidelberg, Germany

**Table 1** Antibody characteristics.

### Double label immunohistochemistry of CD68 and CD163 or DC SIGN

To determine if cells were double positive for CD68 and CD163 or DC SIGN, besides the use of sequential slides, also double labelling was performed. Extensive investigation showed that the combination of CD14 and CD163 or DC SIGN did not give reliable results. Therefore, CD163 or DC SIGN and CD68, a general and a pan-macrophage marker, double labelling was performed. The sections were deparaffinised in xylene followed by alcohol 100%. Blocking was performed with methanol 0.3% H<sub>2</sub>O<sub>2</sub>. The sections were rehydrated and rinsed with PBS. The Tris-HCL buffer (pH8.2, 100 mM) was preheated in a water bath at 97°C. The sections were incubated with the buffer for 30 minutes on 97°C, and cooled down for 45 min. on ice. Thereafter sections were incubated with the first antibody (CD163 or DC SIGN), for 1 hour at room temperature and afterwards rinsed with PBS. The sections were incubated with Envision-HRP anti-mouse (DAKO, North America Inc, USA) for 30 minutes and rinsed with PBS. For 7 minutes at room temperature the sections were incubated with Vector NovaRed (Vector Laboratories Inc, Burlingame, USA) and rinsed with PBS. Then the sections were incubated with the second antibody (CD68) for 1 hour, followed by incubation with Rabbit anti-mouse (DAKO) for 30 minutes, APAAP mouse (DAKO) for 30 minutes and with Vector blue (Vector laboratories Inc, Burlingame, USA) for 25 minutes. In between each step the slides were rinsed with PBS. Finally, the sections were dried and covered with mounting medium (Pertex, Histolab Products, Gothenburg, Sweden).

### Double label immunohistochemistry of CD14 and Flt-1

Double-immunohistochemistry staining of CD14 and Flt-1 was performed using the DAKO Envision G/2 Doublestain system (code K5361) following the manufacturers protocol. Briefly, slides were deparaffinized and hydrated via graded alcohols to demiwater. Heat-induced antigen-retrieval was performed with citrate buffer (pH 6.0) for 20 minutes in a microwave, followed by washing steps in PBS. Endogenous alkaline phosphatase and peroxidase activity was blocked for 5 min by dual endogenous enzyme block. The sections were incubated with primary antibody anti-Flt-1 (dilution 1:250, Santa cruz-316), followed by incubation with Polymer/HRP reagent, using DAB+ as chromogen. Next a blocking step with double stain block reagent was performed. The sections were incubated with the second primary antibody anti-CD14 (dilution 1:200 in 1% BSA/PBS, Novocastra, clone 1F6), afterwards a Rabbit/Mouse LINK was added, followed by incubation with Polymer/AP reagent, using Permanent Red as chromogen. As a control, primary antibodies were replaced with isotype control antibodies to obtain single immunohistochemical staining. Double stained sections were counterstained with haematoxylin.

## Quantification of staining

Equivalent fields containing decidua of sequential sections were digitized blinded by study group (Zeiss Axioskop 40, magnification 200x, Zeiss Axiocam MRc 5 camera, 150x150dpi). For every staining of one placenta a total of 15 pictures of the decidua parietalis and 15 of the basalis were taken (3 locations and 5 pictures per location). Only the decidual stroma was selected for evaluation; irrelevant structures like blood vessels and shadows were digitally removed. Using Image-J software [23], the numbers of positive pixels per area were measured indicating the level of expression. The program is able to identify and measure positive cells by setting a threshold. For every staining a macro was made, predefining the threshold of a positive cell. This threshold was independently defined by two observers. Of the 15 pictures the mean and standard deviation of the number of pixels per area were calculated. The CD163/CD14 ratio and the DC SIGN/CD14 ratio were calculated for every side matched pictures. All analyses were performed blinded for the pregnancy group. Placentas included in the preterm preeclamptic group all showed histological characteristics of preeclampsia (increased syncytial knots, chronic villitis, decidual vasculopathy, thickening of trophoblastic basement membrane, and infarction) [24], blindly observed in H&E staining.

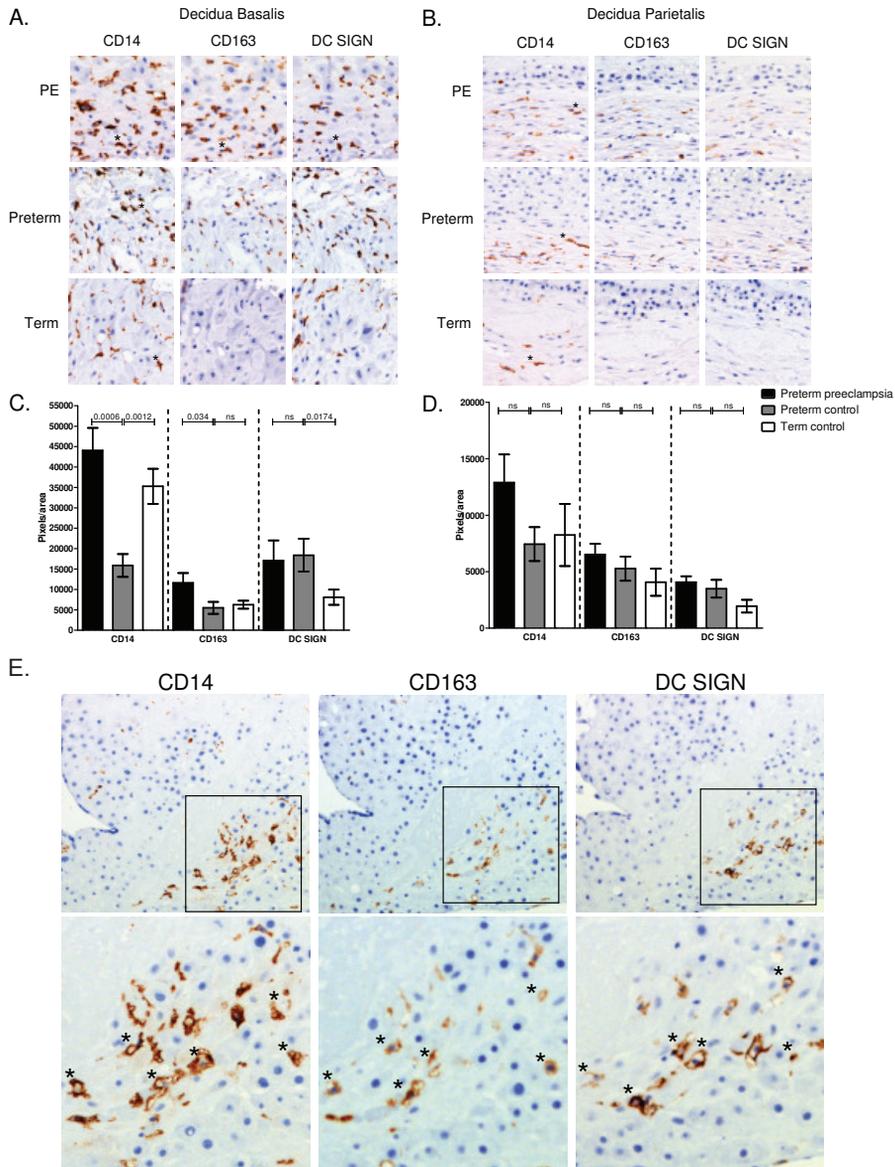
## Statistical analysis

The total amount of pixels per area for every antibody staining was compared between preterm preeclampsia versus preterm control placentas and preterm control versus term control. Ratios (CD163/CD14 and DC SIGN/CD14) were calculated in order to define the amount of CD163+ and DC SIGN+ cells within the macrophage population. Descriptive statistical analysis was performed using Graph Pad Prism (Graph Pad Software Inc.) and SPSS (SPSS Inc 17). A p value of <0.05 was considered statistically significant. The one way ANOVA and the non-parametric Mann Whitney test were used to identify differences between the data.

## Results

### Pilot findings and patient characteristics

In a pilot study of 5 other preterm preeclamptic and term control placentas a difference was found in the level of expression of CD14, CD163 and DC SIGN in preterm preeclamptic and term control. A higher expression rate of CD14 and CD163 and a lower expression rate of DC SIGN was found in decidua basalis of preterm preeclamptic placentas compared with term control placentas (data not shown). Because a difference in gestational age in preterm preeclamptic and term control placentas (40 weeks versus 30 weeks respectively,  $p < 0.05$ ) could have an effect on these outcomes, a preterm control group was collected for the current study. Patient characteristics are shown in Table 2. Patients in the preterm preeclampsia group had a significantly lower gestational age, a higher systolic and a higher diastolic blood pressure ( $p < 0.05$ ) compared with term control and preterm control placentas (Table 2). The gestational age of preterm preeclampsia and preterm control group were 33 and 34 weeks respectively ( $p = 0.033$ ). The decidua of preterm preeclamptic, preterm control and term control placentas all showed positive cells for the used antibodies. Negative control slides were all negative. In general, the average amount of expression for every antigen is higher in the decidua basalis, compared to the decidua parietalis irrespective of the pregnancy group (Figure 1A-D). The staining location of CD14+, CD163+ and DC SIGN+ cells was in general similar at both locations (decidua basalis and parietalis, Figure 1E).



**Figure 1** Photomicrographs of sequential sections stained immunohistochemically for CD14, CD163 and DC SIGN of the decidua basalis (A.) and decidua parietalis (B.) (original magnification x400, positive cells are brown, nuclei are stained blue). The upper row shows the staining in preterm preeclamptic (PE) pregnancies. In the decidua basalis more CD14+ and CD163+ staining is present in the preterm preeclampsia group compared with preterm controls, the amounts of DC SIGN staining do not differ between the preterm preeclampsia group and preterm controls. In the decidua parietalis no significant differences are present. Asterisks indicate examples of positive cells. C and D. Graphs illustrating the amount of positive pixels per area in the decidua basalis (C.) and parietalis (D.) respectively for each antibody in preterm preeclamptic, preterm control or term control placentas. Statistical differences were determined using the non-parametric Mann Whitney test. Values presented as means, the error bars indicate the SEM. E. Photomicrographs of sequential sections stained immunohistochemically for CD14, CD163 and DC SIGN (original magnification x200, positive cells are brown, nuclei are stained blue). In the upper panel the same pattern of staining for the three antigens is visible. The lower panel shows a magnification in which asterisks indicate positive cells for CD14, CD163 and DC SIGN.

	Preeclampsia	Preterm	Term	p value*
Maternal age (years)	31±6	28±2.5	30±2.5	ns
Gestational age (weeks)	33±2	34±0.5	39±1.5	<0.05**
Highest systole (mmHg)	185±10	123±2	126±14	<0.05***
Highest diastole (mmHg)	106±11.5	76±7.5	77±12.5	<0.05***
Gravidity	1	2	1	ns
Parity	0	0	0	ns
Medication	4 x anti-hypertensive	no	no	<0.05****

**Table 2 Patient characteristics.** Plus-minus values are ranges. \* One way ANOVA. \*\*One way ANOVA, followed by t-test showed significant differences between the comparisons of all groups (preeclampsia vs term  $p < 0.0001$ , preeclampsia vs preterm  $p = 0.033$ , preterm vs term  $p = 0.0023$ ). \*\*\*One way ANOVA, followed by t-test showed significant differences between preeclampsia versus term and preeclampsia versus preterm. \*\*\*\*Kruskal-Wallis test.

### Comparison in level of expression of CD14, CD163 and DC SIGN in decidua basalis an parietalis between preterm preeclampsia and preterm control

To compare the phenotype of decidual macrophages of the preterm preeclamptic, term control and preterm control first the expression of the markers CD14 and CD163 were analyzed. The level of expression of CD14 and CD163 was significantly higher in the preterm preeclamptic decidua basalis compared with the decidua basalis of preterm control pregnancies ( $p = 0.0006$  and  $p = 0.034$  respectively, Figure 1C). No significant differences were present in the level of expression of DC SIGN positive cells in the decidua basalis. In the decidua parietalis no significant differences were present between preterm preeclamptic and preterm control pregnancies for CD14, CD163 or DC SIGN (Figure 1A-D).

### Comparison in level of expression of CD14, CD163 and DC SIGN in decidua basalis and parietalis between preterm and term control

As gestational age could have an effect on study outcomes in comparing outcomes of the level of expression in macrophage markers, we also analyzed the differences between the preterm and term control group. Significant differences are present for CD14 and DC SIGN. CD14 expression is significantly lower in the preterm control group compared with the term control group ( $p = 0.0012$ , Figure 1C). CD163 is significantly higher in preterm control group compared with the term control group ( $p = 0.0174$ , Figure 1C). In the decidua parietalis no significant differences were present between preterm and term control pregnancies (Figure 1D).

### The ratio CD163/CD14 is lower and the ratio DC SIGN/CD14 is higher in preterm preeclamptic decidua basalis, when compared with preterm control pregnancies

In general, the sequential stained slides showed a similar staining pattern for CD14, CD163 and DC SIGN although not all CD14+ cells are positive for CD163 or DC SIGN (Figure 1E). To prove that cells were double positive for CD68 and CD163 or DC SIGN next to the use of sequential slide also double labeling was performed. The double staining of CD68 and CD163 or DC SIGN

confirms that some cells which were positive for a general macrophage marker are as well positive for the M2 marker (Figure 2A and B). To examine the natural polarization of decidual macrophages and alterations of the phenotype the CD163/CD14 and DC SIGN/CD14 ratios of subsequent areas were calculated. Although, the individual level of expression of CD163 is higher in preterm preeclamptic decida basalis compared with preterm control (Figure 1C), the number of CD163 positive cells in the fraction of CD14 positive cells (CD163/CD14) was significantly lower in preterm preeclamptic decida basalis compared with preterm control decida basalis ( $p=0.0293$ , Figure 3A). By contrast the level of DC SIGN in the fraction of CD14 positive cells (DC SIGN/CD14) was significantly higher in preterm preeclamptic placentas than in preterm control placentas ( $p<0.0001$ , Figure 3A). As in the decida basalis, in the decida parietalis the ratio DC SIGN/CD14 was significantly higher in preterm preeclamptic and preterm control pregnancies ( $p<0.0001$ , Figure 3B). The ratio CD163/CD14 and ratio DC SIGN/CD14 is significantly higher in decida basalis of preterm controls compared with term controls ( $p=0.0190$  and  $<0.0001$  respectively, Figure 3A). In the decida parietalis the ratio DC SIGN/CD14 is significantly lower in preterm controls compared with term controls ( $p<0.0001$ , Figure 3B).

### CD14+ macrophages are Flt-1+

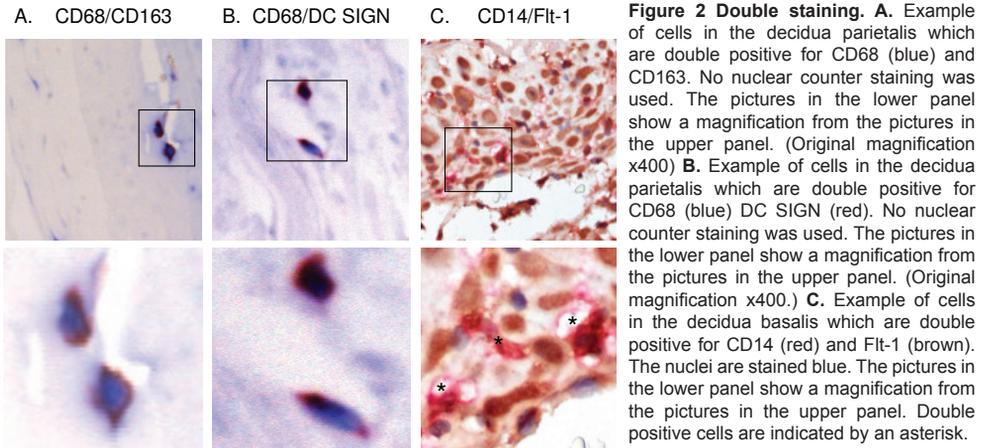
As suggested that decidual macrophages are a possible additional source of sFlt-1 production and thereby they could contribute to the pathogenesis of preeclampsia. Therefore we investigated whether macrophages are positive for Flt-1. Double labeling of CD14 and Flt-1 shows that macrophages in the decida basalis did express Flt-1 (Figure 2C).

### Lower expression of IL-10 in decida parietalis of preterm preeclamptic pregnancies compared with preterm control pregnancies

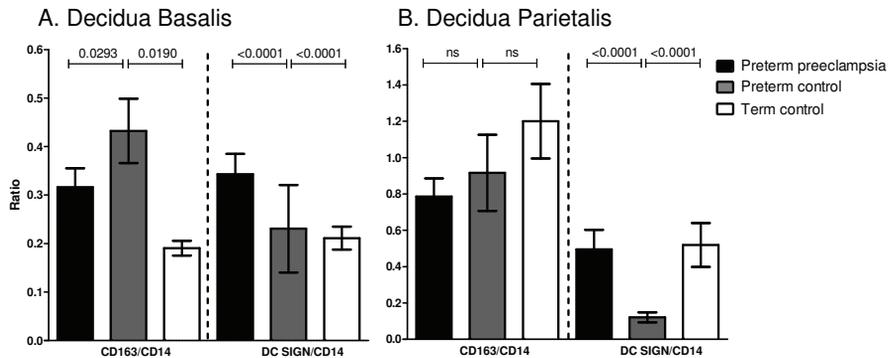
To functionally characterize cells in the decida, immunohistochemical staining of IL-10 was performed on placental tissue. The level of expression of IL-10 in preterm preeclamptic decida parietalis is significantly lower compared with preterm control pregnancies ( $p=0.03$ ). No significant differences were found in the expression of IL-10 in the decida basalis of preterm preeclamptic, preterm control and term control placentas (Figure 4).

## Discussion

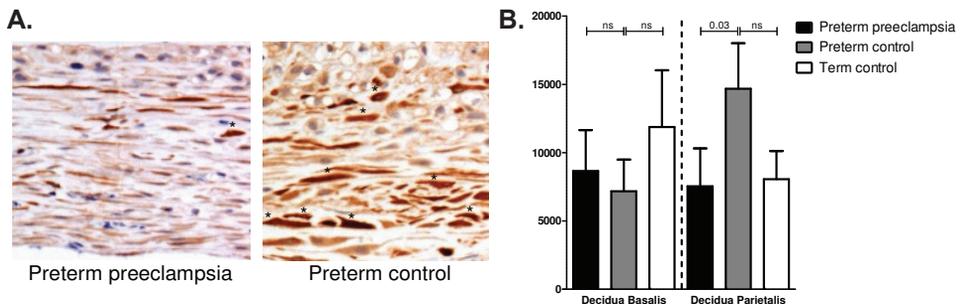
This study investigated the phenotype and natural polarization of decidual macrophages by comparing the myeloid cell markers CD14, CD163 and DC SIGN cells in decida basalis and parietalis of preterm preeclamptic, preterm control, and term control pregnancies using immunohistochemistry and an objective quantification method. We found significantly more CD14+ cells in the decida basalis in preterm preeclamptic pregnancies compared with preterm control pregnancies. In addition the specific M2 marker CD163, was significantly upregulated in the decida basalis in preterm preeclamptic pregnancies compared with preterm control pregnancies. Insight of the functional importance of the phenotypic differences in decidual macrophages is limited by lack of M1 markers, and therefore the M2 ratio of CD163/CD14 was used. In the decida basalis the number of M2 cells (ratio of CD163/CD14) was significantly lower in placentas from preterm preeclamptic pregnancies compared with preterm control pregnancies. The ratio DC SIGN/CD14 was significantly higher in decida basalis and parietalis of preterm preeclamptic pregnancies compared with preterm control pregnancies. In addition to the preterm control group we compared the term control group with the preterm control group. A significantly lower level of expression of CD14 was present in the decida basalis of preterm



**Figure 2 Double staining.** **A.** Example of cells in the decidua parietalis which are double positive for CD68 (blue) and CD163. No nuclear counter staining was used. The pictures in the lower panel show a magnification from the pictures in the upper panel. (Original magnification x400) **B.** Example of cells in the decidua parietalis which are double positive for CD68 (blue) DC SIGN (red). No nuclear counter staining was used. The pictures in the lower panel show a magnification from the pictures in the upper panel. (Original magnification x400.) **C.** Example of cells in the decidua basalis which are double positive for CD14 (red) and Flt-1 (brown). The nuclei are stained blue. The pictures in the lower panel show a magnification from the pictures in the upper panel. Double positive cells are indicated by an asterisk.



**Figure 3 A.** Ratio CD163/CD14 and DC SIGN/CD14 calculated from subsequent pictures. The ratio CD163/CD14 is significantly lower ( $p=0.0293$ ) and the ratio DC SIGN is significantly higher ( $p=0.0001$ ) in preterm preeclamptic decidua parietalis compared with preterm control pregnancies. **B.** The ratios of CD163/CD14 and DC SIGN/CD14 in the decidua parietalis. The ratio CD163/CD14 is not significantly different and the ratio DC SIGN is significantly higher ( $p=0.0001$ ) in preterm preeclamptic decidua parietalis compared with preterm control pregnancies.



**Figure 4 IL-10 results.** **A.** Photomicrographs of sections stained immunohistochemically for IL-10 in preterm preeclamptic and preterm control decidua parietalis (original magnification x400). Asterisks indicate examples of positive cells. **B.** In the decidua basalis no significant differences are present in the amount of IL-10+ cells between preterm preeclamptic, preterm control or term control pregnancies. In the decidua parietalis less IL-10 staining is present in the preterm preeclampsia group compared with preterm controls ( $p=0.03$ ).

control compared with term controls ( $p=0.0012$ ). This indicates that it is important to have a gestational age matched control group when investigating macrophages in preterm preeclamptic pregnancies.

The most abundant differences are found in the decidua basalis, and not in the decidua parietalis, which could be explained by the invasion of trophoblast which occurs in the decidua basalis and not in the decidua parietalis.

Maternal tolerance towards the semi-allogeneic fetus is important for an uncomplicated pregnancy. The decidual cell population consists of several immunologic cells and a disturbance in the distribution of phenotype of these cells may lead to pregnancy complications. Macrophages and DCs are present in the human decidua [6,19,25,26] and an alteration of the phenotype and distribution may be involved in the pathogenesis of preeclampsia [27].

The sequential stained immunohistochemical slides showed that in general CD14+ cells can also be DC SIGN+ and CD163+. Our study confirms earlier reports of predominant polarization to M2 macrophages in the term placenta (reviewed by Nagamatsu et al [28]). The amount of CD14+ or CD163+ cells in the decidua basalis were significantly higher in placentas from preterm preeclamptic pregnancies compared with preterm control pregnancies. Severity of preeclampsia could contribute to this higher number and different functionality of macrophages present in the decidua. Therefore, two placentas from most severe cases of preeclampsia (based on the level of diastolic pressure, amount of proteinuria and gestational age) demonstrated the highest number of cells in the decidua basalis.

The number of M2 macrophages in relation to all macrophages (ratio CD163/CD14) was lower in placentas from preterm preeclamptic pregnancies, compared with preterm control pregnancies. To our knowledge, this is the first study that describes a decrease in M2 in the decidua basalis of preterm preeclamptic pregnancies compared to preterm control pregnancies. We speculate that this lower amount of M2 may contribute to the etiology of preeclampsia. Furthermore, we have shown an increase of the ratio of DC SIGN+ cells in placentas from preterm preeclamptic pregnancies. The phenotypic plasticity of myeloid cells such as DCs and macrophages is substantial and a subset distinction is difficult to make. Only a few markers are known which really make the distinction between macrophages and DCs. Gardner et al [19] already postulated that DC SIGN is present on decidual macrophages but not on decidual DCs. It remains unclear whether this cell subset, also called intermediate antigen presenting cells, is a subset of macrophages or of DCs. CD14+DC SIGN+ cells are reported in other human tissues [29,30], and these cells produce large amounts of proinflammatory cytokines [31]. In line with the study of Gardner et al, our study also shows in general a similar staining pattern between CD14+ and DC SIGN+ cells. The presence of this subset of DC SIGN+ macrophages in the decidua is pregnancy-associated and these cells may play a crucial role for the local immune response. Therefore, alterations in the function and distribution of this cell may result in pathological pregnancies, like preeclampsia which has been shown by Huang et al [32]. Preeclamptic decidua contained an infiltrate of DC SIGN+ cells in contrast to their sparse presence in the decidua of uncomplicated pregnancies. This study also confirms an increased level of DC SIGN expression in preterm preeclamptic decidua compared to preterm control decidua. However, current study relates DC SIGN+ cells with macrophages instead of DCs because of their co-localization and as shown by double staining. In contrast to our study, Scholz et al found no significant differences between preeclamptic and control placentas in the amount of DC SIGN+ cells using immunohistochemistry [33]. However, they found a higher amount of DC SIGN+ cells in placentas from patients who developed HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome. It is possible that our preterm preeclamptic group is more comparable with the HELLP group of the study of Scholz et al. since our study included only very severe preterm preeclamptic patients with deliveries with a gestational age below 34 0/7 weeks.

In addition of the presence macrophage antigens in the decidua, this study investigated the production of IL-10 in placental tissue. During pregnancy IL-10 is an important cytokine, it plays a role in the prevention of placental rejection. Human pregnancy is a type 2 immune state shown by a shift in cytokine production from type 1 to type 2. This balance is different in preeclampsia in which a decrease in IL-10 compared to the pro-inflammatory cytokines is present. IL-10 is secreted by cytotrophoblast and it can suppress an allogeneic immune response *in vitro* [34]. It is possible that IL-10 may be involved in protecting the semi-allogeneic fetus in normal pregnancy [21]. To our knowledge, only one earlier published study performed IL-10 immunohistochemical staining on placental tissue [22]. Hennesy *et al.* showed a change in IL-10 immunolocalization in term placentas from women with preeclampsia compared to those with a normal pregnancy outcome. They showed a general decrease in cytoplasmic trophoblast villi IL-10 content in preeclamptic pregnancies. Additionally, a decrease in IL-10+ trophoblast cells located in the decidual tissue was present. A lower level of IL-10 in the decidua basalis suggests an impaired protective mechanism of the mother toward the allogeneic fetus in preeclampsia.

Our digital analysis shows that the number of IL-10 positive cells is lower in the decidua parietalis of preeclamptic pregnancies compared to preterm pregnancies. This indicates that there is a difference in defense mechanism between the decidua parietalis and basalis. The decidua parietalis contacts the non-invading trophoblast of the chorion and the decidua basalis interacts with invading villous trophoblast. It seems that the contact between the chorion in the decidua parietalis in preterm pregnancies synthesizes the trophoblast cells to produce IL-10, which does not appear in preeclamptic decidua parietalis. Since this study showed a lower amount of positive IL-10 cells in the decidua parietalis of preeclamptic pregnancies compared with preterm pregnancies, we speculate that a high level of IL-10 is necessary to maintain pregnancy without complications, and that a down regulation of IL-10 produced by the decidua parietalis is a permissive condition for the development of preeclampsia.

Recently, it has been shown in chronic kidney disease that monocytes may be a possible source of sFlt-1 [35]. Increase of sFlt-1 leads to endothelial dysfunction and increased levels have been found in patients with preeclampsia [10,36]. Double labeling immunohistochemical staining of CD14+ and Flt-1 shows that macrophages in the decidua basalis are positive for Flt-1. Since we found an increase of the amount of CD14+ cells in preeclamptic decidua basalis compared with preterm decidua basalis ( $p=0.0006$ ) it is possible that decidual macrophages are responsible for the increased sFlt-1 production which may contribute to the etiology of preeclampsia.

Tolerance of the genetically foreign fetus by the maternal immune system fetus is a complex phenomenon and remains to be elucidated. Multiple mechanisms are involved in maintaining the pregnancy. Localized secretion of immunoregulatory cytokines may prevent immune rejection of the placenta. In addition, the presence of immunomodulatory cells may be important in dampening an inflammatory immune response. Preeclampsia is a state in which the immune system has to work harder to maintain pregnancy. Alterations in immunomodulatory cells in the decidua basalis and parietalis of preterm preeclamptic pregnancies compared to control pregnancies may contribute to the etiology of preeclampsia. The question is whether alterations in the immune system lead to the pathogenesis of preeclampsia or its prevention in subsequent pregnancies.

In conclusion, present study shows that macrophages can be DC SIGN+ as well as CD163+ based upon the double staining and based on the similar staining pattern of these antigens. An increase of CD163+ cells in preterm preeclamptic placentas was found compared with preterm control placentas. However, the total amount of CD14+ cells is also increased in preterm preeclamptic placentas compared with preterm control placentas. The amount of CD163+ cells in the fraction of CD14+ cells is lower in preterm preeclamptic placentas compared with preterm control placentas. Furthermore, this study found an increase in DC SIGN/CD14 myeloid cells in the decidua parietalis and basalis of preterm preeclamptic pregnancies compared with preterm control pregnancies.

This study suggests that further investigation of the distribution and phenotype of macrophages is possibly relevant for further understanding the immunology at the fetal-maternal interface.

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