## BASIC SCIENCE: OBSTETRICS

# Plasma of pregnant and preeclamptic women activates monocytes in vitro

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**OBJECTIVE:** The objective of the study was to test the hypothesis that factors circulating in the plasma of pregnant women and women with preeclampsia activate monocytes.

**STUDY DESIGN:** Blood samples were taken from patients with early-onset severe preeclampsia (n = 9), healthy pregnant women (n = 9), and healthy nonpregnant women (n = 9). A monocytic cell line was incubated with the plasma for 4, 16, and 24 hours. After the incubation, reactive oxygen species (ROS) production and intercellular adhesion molecule (ICAM)-1 expression (protein and messenger ribonucleic acid) were measured.

**RESULTS:** Plasma of both pregnant women and women with preeclampsia, as compared with plasma from nonpregnant women, in-

creased the mean channel brightness (MCB) of ROS after 4 hours of incubation, whereas only plasma of pregnant women increased the percentage of cells producing ROS (after 4 and 24 hours of incubation). Plasma of pregnant women and women with preeclampsia up-regulated the percentage of ICAM-1–expressing cells after 4 hours and down-regulated the percentage of ICAM-1–expressing cells and MCB after 24 hours.

**CONCLUSION:** Plasma of both pregnant women and women with preeclampsia activated monocytes in vitro.

**Key words:** intercellular adhesion molecule, monocytes, preeclampsia, plasma, pregnant, reactive oxygen species

Preeclampsia is a disorder unique to pregnancy and characterized by hypertension and proteinuria occurring in the second half of pregnancy. Worldwide it is a leading cause of morbidity and mortality in pregnancy. Although the pathogenesis of the disease is not completely understood, a generalized inflammatory response is suggested to play an important role in the pathogenesis.[1] The factor(s) responsible for activating the inflammatory response are unknown. It has, however, been suggested that reduced vascular trophoblast invasion and insufficient remodeling of the spiral arteries results in abnormal placentation and placental underperfusion,[2] This may lead to the production of substances from the dysfunctional placenta, which may systemically activate inflammatory cells, such as monocytes, granulocytes, and endothelial cells. Many substances have been proposed, such as cytokines[3] and syncytiotrophoblast microvillous membrane fragments.[4]

The generalized inflammatory response of preeclampsia is characterized by activated endothelial cells[5] and activated in-

flammatory cells.[6] Various studies have demonstrated that circulating leukocytes are activated in preeclampsia.[6-8] For instance, up-regulation of the activation marker CD11b was shown on both monocytes and granulocytes of women with preeclampsia as compared with normal pregnant women.[6] Moreover, both monocytes and granulocytes produce increased amounts of oxygen free radicals in women with preeclampsia as compared with normal pregnant women.[6]

There is also a lot of evidence for activation of endothelial cells in preeclampsia because increased levels of von Willebrand factor,[9] endothelin,[9] soluble E-selectin, and soluble vascular cell adhesion molecule (sVCAM)-1[5] have been found in plasma from preeclamptic patients. There is, however, no direct evidence for endothelial cells activation in preeclampsia. A recent study by Donker et al[10] showed no proinflammatory activation of endothelial cells in abdominal fat, fascia, and myometrium biopsies of patients with preeclampsia, whereas Lyall at al[11] also demonstrated no differences in proinflammatory adhesion molecule expression between myo-

**TABLE 1**
**Characteristics of patients and controls**

| | Preeclampsia (n = 9) (mean [SEM]) | Pregnant controls (n = 9) | *P* value |
|---|---|---|---|
| Maternal age (y) | 29.3 (1.97) | 31.1 (1.04) | NS |
| Caucasian | 9 | 9 | NS |
| Nulliparity | 9 | 9 | NS |
| Blood sampling | | | |
|    Gestational age (wks) | 30.6 (1.2) | 31.3 (2) | NS |
|    Systolic blood pressure (mm Hg) | 155 (3) | 118 (3.7) | < .01 |
|    Diastolic blood pressure (mm Hg) | 99 (3.3) | 70 (2.6) | < .01 |
|    Proteinuria (g per 24 h) | 6.2 (1.2) | n.d. | |
|    Uric acid (mM) | 0.42 (0.03) | 0.24 (0.02) | < .01 |
|    Platelet count ($10^9$/L) | 169.8 (39) | 242 (13) | < .01 |
| Delivery | | | |
|    Gestational age (wks) | 31.3 (1.11) | 38.99 (0.6) | < .01 |
|    Birthweight (g) | 1421 (182) | 3744 (257) | < .01 |

*n.d.*, not determined.

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metrial vessels of normal pregnant and women with preeclampsia. This may suggest that endothelial cell activation in preeclampsia may be restricted to specialized vascular beds, such as in the kidney or liver.

Not only preeclampsia but also normal pregnancy is associated with an activated inflammatory response, albeit much less than in patients with preeclampsia.[6,7] Activation of monocytes and granulocytes has been shown by increased expression of the adhesion molecules CD11b and CD64[6] and intercellular adhesion molecule (ICAM)-1[12] as well as an increased basal production of reactive oxygen species[6] and cytokine production.[13] It seems unlikely that in the inflammatory response of normal pregnancy, endothelial cells are involved because in normal pregnancy, no increased soluble ICAM-1[14,15] or sVCAM-1[15] was found. It may be suggested that the placenta is also involved in activating the inflammatory cells during normal pregnancy because, for instance, STBMs have also been found in normal pregnancy.[4] Also, other factors produced by the placenta may be involved, such as cytokines.[16] The reason that inflammatory cells are activated during normal pregnancy remains unknown, although it may be suggested that it is a compensatory response for the change in Th1/Th2 balance toward Th2 during normal pregnancy.[17]

The present study was set up to test the hypothesis that plasma factors of both women with preeclampsia and pregnant women activate monocytes and that plasma factors of women with preeclampsia induce higher monocyte activation as compared with plasma of normal pregnant women. Therefore, we used a monocyte cell line (monomac-6 cells), which was incubated for various intervals with plasma of women with preeclampsia, gestational age–matched normal pregnant women and age-matched nonpregnant women. The activational state of monocytes was measured using 2 parameters, ICAM-1 expression and oxygen-free radical production. Increased expression of ICAM-1 on monocytes has been shown after activation of these cells with various stimuli (ie, lipopolysaccharide [LPS]).[18] Moreover, monocyte ICAM-1 expression was shown to be increased in monocytes of pregnant women as compared with monocytes of nonpregnant women.[12,19] It is well known that on activation of monocytes, they start producing reactive oxygen species (ROS),[20] whereas monocyte ROS production is increased during pregnancy and preeclampsia.[6] Both ICAM-1 expression and ROS production were measured using flow cytometry. ICAM-1 messenger ribonucleic acid (mRNA) expression was measured using real-time reverse transcription–polymerase chain reaction.

## MATERIALS AND METHODS
### Patients
This study was approved by the Medical Ethics Committee of the University Medical Center Groningen. Written informed consent was obtained from all participants. Preeclampsia was defined according to the standards of the International Society for the Study of Hypertension in Pregnancy: a diastolic blood pressure of at least 90 mm Hg on 2 or more consecutive occasions, each more than 4 hours apart, and proteinuria of more than 300 mg per 24 hours. Both should develop after 20 weeks of gestation and return to normal values within 3 months after delivery.

Women who had been diagnosed with a preexisting hypertension, diabetes mellitus, vasculitis, chronic renal disease, autoimmune disease, or malignancy and women who had undergone recent trauma or surgery were excluded from the study. For both the normal pregnancy and preeclamptic group, only singleton pregnancies were included. All patients with preeclampsia (n = 9) had an early-onset development of the disease and participated between 25 and 33 weeks of gestation and at the time of blood sampling had not been treated with corticosteroids.

Controls were healthy pregnant women without hypertension (diastolic less than 90 mm Hg and systolic less than 135 mm Hg) (n = 9). They were matched with women with preeclampsia for age (± 6 years) and gestational age at blood sampling (± 2 weeks). Nonpregnant women (n = 9), recruited from our hospital staff, were matched with women with preeclampsia and normal pregnant women for age. The Table summarizes clinical and laboratory characteristics of the patients and controls.

## Collection and preparation of plasma samples

Maternal blood samples of both pregnant women and women with preeclampsia were collected during routine blood sampling during pregnancy/preeclampsia. Blood samples were drawn from the antecubital vein into 10 mL tubes containing lithium heparin (Venoject, Terumo Europe NV, Leuven, Belgium). Samples were immediately stored at 4°C and centrifuged within 1 hour. They were centrifuged at 130 g for 10 minutes at 4°C; subsequently the plasma was centrifuged at $700 \times g$ for 10 minutes. The platelet poor plasma was frozen in aliquots at −80°C.

## Monocyte cell cultures

In this study we used the monocyte cell line (monomac-6 [MM6]), which is a monocytic cell line, showing phenotypic and functional features of mature monocytes.[21] The reason for using this cell line, rather than isolated peripheral blood monocytes, is the fact that isolation of monocytes from peripheral blood already results in activation of the monocytes.[22,23] The MM6 cell line was cultured in culture flasks in RPMI 1640 medium, supplemented with 10% fetal calf serum, 100 nM sodium pyruvate, 200 nM glutamate, 0.05 M $\beta$-mercaptoethanol (BME), 10 mg/mL gentamicin, and 2.12 $\mu$g/mL Fungizone. Cells were diluted 1:5 every 3-4 days. Only cells that were diluted 3-4 days before and that were at a concentration of about $1 \times 10^6$ cells/mL were used for the experiments.

## Monocyte incubation conditions

Before the start of the experiments, MM6 cells were centrifuged at 1800 rpm for 10 minutes and brought at a concentration of $2 \times 10^6$ cells/mL.

## Experiment 1: LPS responsiveness of MM6

We first determined the ICAM-1 expression and ROS production of the MM6 cells after a strong proinflammatory stimulus (ie, LPS). Therefore, MM6 cells ($2 \times 10^5$/mL) were stimulated with LPS (concentrations ranging from 8 $\mu$g/mL to 0.1 pg/mL) at 37°C and 5% $CO_2$ (n = 4). After incubation for 4 hours, we measured oxygen-free radical production

(Phago Burst Orpegen; Orpegen Pharma, Heidelberg, Germany) using flow cytometry as described in "Flow Cytometry"; pilot studies showed that production of ROS by monocytes was most prominent between 1 and 4 hours of incubation. For ICAM-1 expression, MM6 cells were incubated with LPS for 4 hours (n = 4) and 24 hours (n = 5). After incubation cells were labeled with ICAM-1 (mouse antihuman ICAM-1–fluorescein isothiocyanate [FITC] labeled, IQ Products, Groningen, The Netherlands) or isotype control (IQ Products) as described in the following text and measured by flow cytometry as described in "Flow Cytometry".

## Experiment 2

To study the effect of factors in the plasma of pregnant women and women with preeclampsia on monocyte oxygen-free radical production, MM6 cells ($2 \times 10^5$/mL) were incubated in RPMI 1640 supplemented with gentamicin and fetal calf serum (FCS) (9 %) and 15 % plasma of pregnant women (n = 9), women with preeclampsia (n = 9), or nonpregnant women (n = 9) or 15% FCS (for control) for 4, 16, or 24 hours in 12-well plates. Thereafter oxygen-free radical production was measured as described in "Flow Cytometry". In each separate experiment, cells were incubated with plasmas from a woman with preeclampsia, gestational age matched with plasma from a normal pregnant woman and age matched with plasma from a nonpregnant woman.

## Experiment 3

In this third experiment, we investigated the effects of plasma's from the 3 groups on MM6 ICAM-1 expression on the protein and mRNA level. Therefore, MM6 ($2 \times 10^5$/mL) were incubated in RPMI 1640 supplemented with gentamicin and FCS (9%) and 15% plasma from nonpregnant (n = 9), pregnant (n = 9), and women with preeclampsia (n = 9) or 15% FCS (for control) for 4, 16, and 24 hours. After incubation, two thirds of the cells were used to measure ICAM-1 mRNA (as described below), the other one third for measuring ICAM-1 protein

expression using flow cytometry as described below.

## Flow cytometry

### Oxygen-free radical production

Oxygen-free radical production was measured using the Burst test (Phagoburst) from Orpegen Pharma. In brief, immediately following incubation of the cells with plasma for 4, 16, or 24 hours, the wells were incubated for 10 minutes with the fluorogenic substrate dihydrorhodamine 123 according to the manufacturer's protocol. Thereafter the reaction was stopped and cells were fixed by adding 2 mL lysing solution supplied in the kit for 20 minutes. Then cells were spun down (5 minutes, 1800 rpm) and washed once with washing solution supplied by the kit, and finally 300 $\mu$L of washing solution was added. Cells were kept on ice and measured by flow cytometry (Calibur, BD Biosciences, Franklin Lakes, NJ) within 30 minutes. Percentage positive cells as well as mean channel brightness of the positive cells (as a measure of total amount of oxygen-free radicals produced) was calculated using Winlist 6.0 (Verity Software House, Topsham, ME).

### ICAM-1 expression

Immediately following incubation with plasma for 4, 16, or 24 hours, one third (about 60,000 cells) of the cells in each well was used for measuring ICAM-1 protein expression. These cells were divided over 2 5 mL tubes and spun down (5 minutes, 1800 rpm). The supernatant was discarded, and 1 of the 2 tubes was incubated with FITC-labeled anti–ICAM-1, and the other tube was incubated with the isotype control for 30 minutes at room temperature in the dark. Then 2 mL washing buffer (phosphate-buffered saline [PBS] with 5% bovine serum albumin and 0.1% sodium azide) was added to each tube, after which the tubes were centrifuged for 5 minutes at 1800 rpm. The supernatant was discarded and a paraformaldehyde (PFA) solution was added to the cells (0.5% PFA in PBS). Cells were kept at 4°C in the dark until ICAM-1 expression was measured by flow cytometry within 24 hours. During the measurement, 10,000 cells were collected. Winlist 6.0
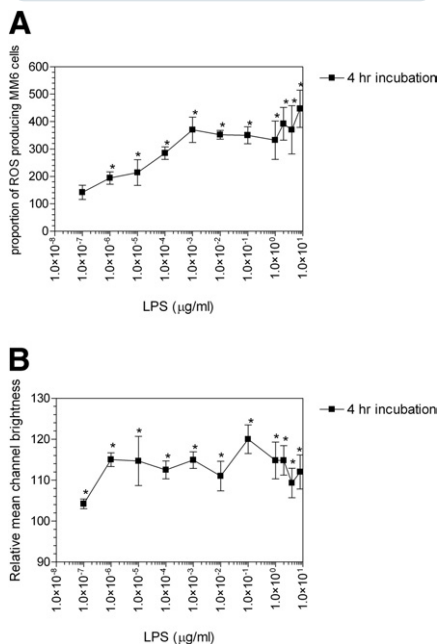
**A**



**B**



**A**, Percentage ROS-producing MM6 cells, expressed as percentage of unstimulated cells, after stimulation for 4 hours with increasing LPS concentrations. **B**, Mean channel brightness of ROS-producing MM6 cells, expressed as percentage of unstimulated cells, after stimulation for 4 hours with increasing LPS concentrations. *Asterisk* denotes significantly increased as compared with unstimulated cells (Wilcoxon, $P <$ .05).

was used to calculate the percentage of positive cells as well as mean channel brightness. Therefore, the monomac cells were gated in a forward-sidescatter plot and copied to a histogram. The isotype control of each sample was used to set the gate so that 99% of the cells were negative. This gate was then copied to the sample stained for ICAM-1, and percentage positive cells and mean channel brightness were determined.

## RNA isolation

The remainder of the cells of the ICAM-1 expression experiments (about 140,000 cells) were used for measuring ICAM-1 mRNA. Following incubation with plasma from the 3 groups for 4, 16, or 24 hours, we isolated RNA from each sample using the absolutely RNA Microprep kit (Stratagene, La Jolla, Calif.) according to the manufacturer's protocol. The quality of the RNA was checked by standard gel electrophoresis, and quantification was performed using the Nanodrop (Thermo Fisher Scientific, Wilmington, Del.). Total RNA was consistently found to be pure and intact.

## Quantitative real-time RT-PCR experiments

For real-time RT-PCR analysis, cellular RNA was converted into first-strand complementary deoxyribonucleic acid (cDNA) using Superscript III RNase H-reverse transcriptase (Invitrogen, Breda, The Netherlands) with oligo dT primers (Promega, Leiden, The Netherlands) and RNase inhibitor (RNaseOUT, Invitrogen). After the reverse transcriptase reaction, the cDNA was diluted to 4 ng/$\mu$L. Real-time PCR amplifications were performed on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Applera Nederland, Nieuwekerk aan de Ijssel, The Netherlands).

We used specific primers sets for ICAM-1 (forward primer: 5′CACAGTCACCTATGGCAACG; reverse: 5′TTCTTGATCTTCCGCTGGC) and $\beta$-actin (forward primer: 5′GCAAATGCTTCTAGGCGGACTAT; reverse primer: 5′CATCTTGTTTTCTGCGCAAGTT and Cybrgreen (Cybr Green PCR master mix, Applied Biosystems).

The real-time PCR system included the following: PCR cycling conditions of 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute, and then melting curve analysis was performed over the range of 55-95°C by monitoring iQ SYBR green fluorescence (Applied Biosystems, Foster City, Calif.) with increasing temperature (0.5°C increment changes at 10 second intervals). Specific products were determined as clear single peaks at their melting curves. All sample measurements were performed in triplicate. Sample cycle threshold (Ct) values were determined from plots of relative fluorescence units vs PCR cycle number during exponential amplification so that sample measurement comparisons were possible. The Ct value for ICAM-1 expression was normalized to $\beta$-actin expression (delta Ct = $Ct_{[\beta\text{-actin}]}$ − $Ct_{[ICAM\text{-}1]}$). The delta delta Ct value of the various incubation times of pregnant and preeclamptic plasma vs plasma from nonpregnant women was evaluated and fold change vs nonpregnant plasma calculated.

## Statistics

All results are expressed as mean ± SEM. For the first experiment (LPS-induced ROS production and ICAM-1 expression), the unstimulated cells were set to 100%. The stimulated cells were related to the unstimulated cells. Wilcoxon signed rank test was used to show significant differences between unstimulated and stimulated cells. Results were considered significant if $P <$ .05.

For experiments 2 and 3, results for nonpregnant women were set to 100%; the results for pregnant women and women with preeclampsia were related to the results for nonpregnant women, and results for pregnant women were compared with results for women with preeclampsia. Differences between groups were tested with the Wilcoxon signed rank test, with significance level of $P <$ .05.

## RESULTS
### Experiment 1: LPS responsiveness of MM6

Stimulation of MM6 cells with LPS for 4 hours with increasing LPS concentrations resulted in an increased percentage of MM6 producing ROS (Figure 1, **A**). The percentage of cells producing ROS increased dose dependently with increasing LPS concentrations. The mean channel brightness of the positive cells (Figure 1, **B**) was increased by LPS stimulation from 1 × $10^{-7}$ $\mu$g/mL and higher. At these concentrations the mean channel brightness did not vary with the dose of LPS.

Stimulation of MM6 cells with similar concentrations of LPS for 4 and 24 hours also resulted in an increase in percentage ICAM-1–positive cells (Figure 2, **A**) as well as in increased mean channel brightness for ICAM-1 (Figure 2, **B**). Figure 2A shows that the percentage of ICAM-1–positive cells was dose depen-

**Effect of LPS on ICAM-1 expression by MM6 cells**
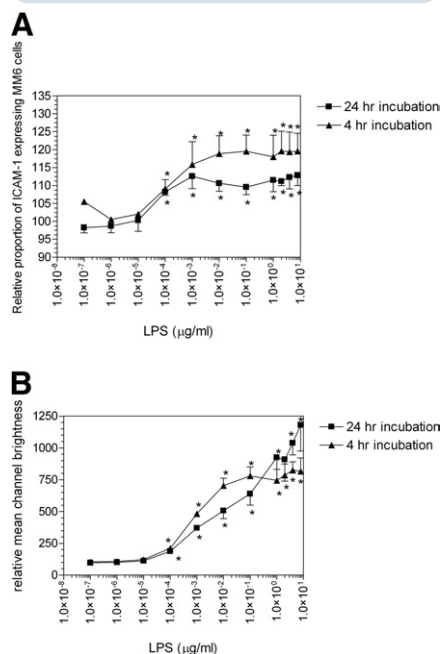


**A**, Percentage ICAM-1–positive MM6 cells, expressed as percentage of unstimulated cells, after incubation with increasing concentrations of LPS for 4 hours (*triangles*) and 24 hours (*squares*). **B**, Mean channel brightness of ICAM-1–positive MM6 cells, expressed as percentage of unstimulated cells, after incubation with increasing concentrations of LPS for 4 hours (*triangles*) and 24 hours (*squares*). *Asterisk* denotes significantly increased as compared with unstimulated cells (Wilcoxon, $P < .05$).

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dently increased but was already maximal when stimulated with $1 \times 10^{-3}$ $\mu$g/mL (24 hours) or $1 \times 10^{-2}$ $\mu$g/mL (4 hours) of LPS. Also the intensity of staining (Figure 1, **B**) was dose dependently increased by LPS stimulation from a dose of $1 \times 10^{-4}$ $\mu$g/mL and higher.

## Experiment 2: ROS production after incubation of MM6 with plasma of nonpregnant, pregnant, and women with preeclampsia

**ROS production:** Figure 3, **A** shows the percentage ROS-producing MM6 cells after 4, 16, or 24 hours' incubation with plasma from nonpregnant women, pregnant women, and women with pre-
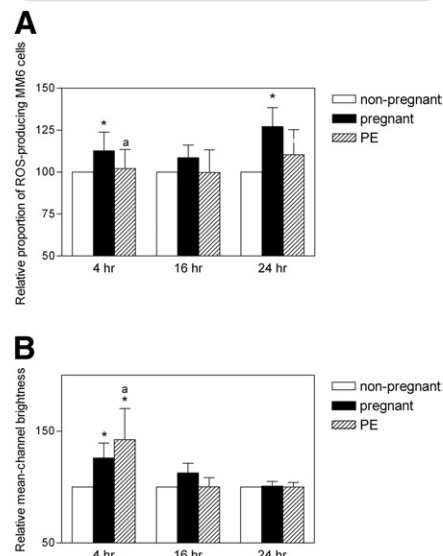
eclampsia, expressed as a percentage of nonpregnant plasma. The mean percentage of cells producing ROS after 4 hours' incubation with plasma from nonpregnant women is $15.4 \pm 3.12$. This percentage did not vary with incubation time and was not significantly different from cells incubated with FCS for control (mean: $14.9 \pm 3.87$) (not shown in the figure). It can be seen from the figure that incubation of MM6 cells with plasma from healthy pregnant women significantly increased the percentage of cells producing ROS after 4 and 24 hours of incubation as compared with plasma of nonpregnant women.

Percentage ROS producing MM6 4 hours after incubation with plasma from preeclamptic women was significantly decreased as compared with plasma from normal pregnant women and not significantly different from plasma from nonpregnant women. After 24 hours' incubation with plasma of women with preeclampsia, percentage ROS-producing MM6 cells did not differ from both normal pregnant women and nonpregnant women. The mean channel brightness (ie, the total amount of ROS per cell) was significantly increased after 4 hours of incubation with plasma from pregnant and even further with plasma from women with preeclampsia as compared with plasma from nonpregnant women (Figure 3, **B**).

## Experiment 3: ICAM-1 expression after incubation of MM6 with plasma of nonpregnant women, pregnant women, and women with preeclampsia
**Protein expression**

Figure 4, **A** shows the percentage of cells expressing ICAM-1 following 4, 16, or 24 hours' incubation with plasma from nonpregnant women, normal pregnant women, and women with preeclampsia. Results are expressed as a percentage of nonpregnant women. Plasma from nonpregnant women slightly but significantly increased the percentage of cells expressing ICAM-1 as compared with incubation with FCS ($89.15 \pm 2\%$ vs $85.4 \pm 3.12\%$, respectively; results not shown). There was no significant effect of time of incubation on MM6 cell

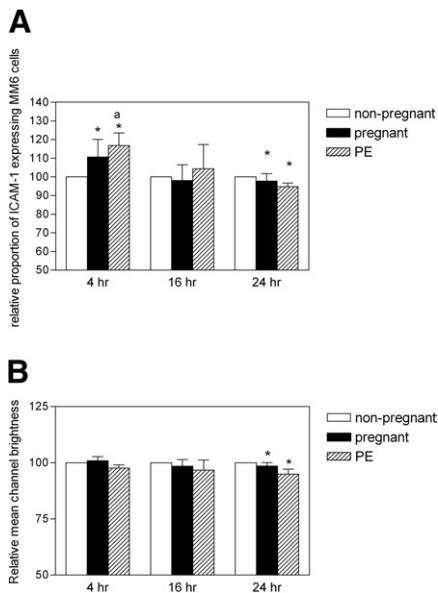**Effect of plasma from nonpregnant, pregnant and preeclamptic women on ROS production by MM6 cells**



**A**, Percentage ROS-producing MM6 cells after 4, 16, and 24 hours' incubation with plasma from nonpregnant women (*open bars*), pregnant women (*black bars*), and women with preeclampsia (*striped bars*). Results are expressed as percentage of nonpregnant plasma. **B**, Mean channel brightness of ROS-producing MM6 cells after 4, 16, and 24 hours' incubation with plasma from nonpregnant women (*open bars*), pregnant women (*black bars*), and women with preeclampsia (*striped bars*). Results are expressed as percentage of nonpregnant plasma. *Asterisk* denotes significantly increased as compared with plasma from nonpregnant women (Wilcoxon, $P < .05$). The letter *a* denotes significant change as compared with plasma from pregnant women (Wilcoxon, $P < .05$).

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ICAM-1 expression after incubation with plasma from nonpregnant women.

Plasma from both normal pregnant women and women with preeclampsia significantly increased the percentage of cells expressing ICAM-1 after 4 hours of incubation as compared with plasma from nonpregnant women (Figure 4, **A**); percentage of cells expressing ICAM-1 after incubation with plasma from women with preeclampsia was signifi-

## Effect of plasma from nonpregnant, pregnant and preeclamptic woman on ICAM-1 protein expression by MM6 cells



**A**, Percentage ICAM-1 positive MM6 cells after 4, 16, and 24 hours' incubation with plasma from nonpregnant women (*open bars*), pregnant women (*black bars*), and women with preeclampsia (*striped bars*). Results are expressed as percentage of nonpregnant plasma. **B**, Mean channel brightness of ICAM-1 positive monomac-6 cells after 4, 16, and 24 hours' incubation with plasma from nonpregnant women (*open bars*), pregnant women (*black bars*), and women with preeclampsia (*striped bars*). Results are expressed as percentage of nonpregnant plasma. *Asterisk* denotes significantly increased or decreased as compared with plasma from nonpregnant women (Wilcoxon, *P* < .05). The letter *a* denotes significant change as compared with plasma from pregnant women (Wilcoxon, *P* < .05).

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cantly increased as compared with plasma from normal pregnant women. After 24 hours' incubation, however, the percentage of ICAM-1 positive cells was significantly decreased following incubation with plasma from pregnant women and women with preeclampsia as compared with plasma from nonpregnant women. There was no significant difference between plasma from women with preeclampsia and normal pregnant women.

The mean channel brightness of ICAM-1 (Figure 4, **B**) was significantly decreased following incubation with plasma from pregnant women and women with preeclampsia for 24 hours as compared with plasma from nonpregnant women, with no significant difference between plasma from normal pregnant women and women with preeclampsia. No difference in intensity of staining was found between FCS incubations and plasma from nonpregnant women ( 85.2 ± 6.7 vs 81.5 ± 5.08, respectively), and no effect of time of incubation was observed following incubation with plasma from nonpregnant women (results not shown).
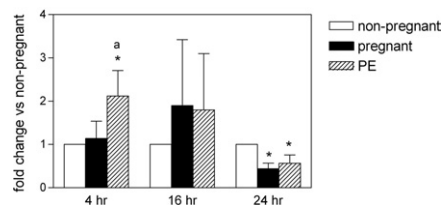
### mRNA expression
Figure 5 shows the ICAM-1 mRNA expression of MM6 cells following incubation with plasma from the 3 groups of women for 4, 16, and 24 hours. The figure shows that ICAM-1 mRNA expression was increased after 4 hours of incubation with plasma from women with preeclampsia as compared with plasma from nonpregnant women and normal pregnant women. After 24 hours of incubation, however, the ICAM-1 mRNA was decreased after incubation with plasma from pregnant women and women with preeclampsia as compared with plasma from nonpregnant women, with no difference between plasma from women with preeclampsia and normal pregnant women.

### COMMENT
In the present study, we tested the hypothesis that factors circulating in the plasma of pregnant and especially preeclamptic women active monocytes. This is the first study evaluating the effect of plasma of pregnant women and women with preeclampsia on monocyte activation. We showed that plasma from both normal pregnant women and women with preeclampsia increased ROS production and ICAM-1 expression, suggesting activation of this monocyte cell line. These results are in agreement with the fact that circulating

## Effect of plasma from nonpregnant, pregnant, and preeclamptic women on ICAM-1 mRNA expression by MM6 cells



ICAM-1 mRNA expression of monomac-6 cells after 4, 16 and 24 hour incubation with plasma from nonpregnant women (*open bars*), pregnant women (*black bars*) and preeclamptic women (*striped bars*). Results are expressed as fold change vs nonpregnant. *Significantly increased or decreased as compared with plasma from nonpregnant women (Wilcoxon, *P* < .05). [a]Significantly increased as compared with plasma from pregnant women (Wilcoxon, *P* < .05).

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monocytes in both pregnant women and women with preeclampsia are activated and produce increased amounts of ROS and show increased expression of ICAM-1.[6,12,19] Thus, factors are present in the plasma of both pregnant women and women with preeclampsia that are able to activate this monocyte cell line. Although it seems likely that such factors arise from the placenta, the nature of these factors is unknown at this time and is the subject of further research.

In the present study, we used a monocyte cell line, MM6. This is a monocyte cell line with characteristics of mature circulating monocytes.[21] The reason for choosing to work with a cell line, rather then with isolated peripheral monocytes, is the fact that the isolation procedure of monocytes activates the monocytes.[22,23] Therefore, working with isolated peripheral monocytes does not allow us to study the effect of plasma on resting monocytes.

In our first experiment, we studied the effect of a strong monocyte activator, LPS, on the MM6 cells. We showed that LPS incubation strongly increased both the pro-

duction of ROS and the expression of ICAM-1 dose dependently. This is in line with the well-known effect of LPS on monocytes,[18,20] suggesting that this cell line indeed responds like mature monocytes and that these 2 markers (ROS production and ICAM-1 expression) were excellent markers to use in our study into the activating effect of plasma from pregnant women and women with preeclampsia on monocytes.

Because we used a dose range of LPS from very low to maximal in this first experiment, the results of this experiment were also used to give an indication of the magnitude of the response of the MM6 cells to plasma. In the present study, we used plasma of patients with severe early-onset preeclampsia. These patients were chosen because they showed the most severe disease, which is most likely of placental origin.[24] We expect the most prominent effect of plasma from these patients, compared with a milder form of the disease.

ROS production by monocytes is due to activation of the NADPH-oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) system.[25] Activation of this NADPH oxidase does result in production of ROS for several hours, up to 24 hours.[25] The peak ROS release (oxidative burst) occurs much earlier (ie, between 0 and 4 hours).[25] This respiratory burst is the first event in full-blown activation of monocytes, which can be induced by various monocyte activating agents (ie, LPS, zymosan, N-formyl-L-methionyl-L-leucyl-L-phenylalanine, or cytokines).[20,25] After incubation of MM6 cells with plasma from both pregnant women and women with preeclampsia, the most prominent effect on ROS production was the increase of mean channel brightness (reflecting the mean production of ROS per cell) after 4 hours of incubation as compared with plasma from nonpregnant women. This increase in mean channel brightness after incubation with plasma for 4 hours is similar or higher than the increase induced by the maximal dose of LPS in experiment 1. Because LPS is a strong activator of monomac cells, this may indicate that the putative plasma factor(s) is a strong activator.

The fact that plasma of women with preeclampsia induced significantly more ROS production by monocytes as compared with plasma from normal pregnant women is in line with previous reports,[6,26] which showed that basal ROS production is increased in monocytes of women with preeclampsia vs monocytes of normal pregnant women. This may suggest that different activating factors are present in preeclamptic vs normal pregnant plasma. Alternatively, increased amounts of identical factors may be present in the plasma of women with preeclampsia as compared with normal pregnant women.

Also, the difference in the percentage of MM6 cells producing ROS may suggest the existence of different factors in the plasma of both groups. After 4 and 24 hours of incubation, only plasma of pregnant women, not women with preeclampsia, increased the percentage of cells producing ROS. As compared with LPS stimulation, this increase in the percentage of ROS-producing cells is very slight (up-regulation by plasma of 112% and 127% [after 4 and 24 hours' incubation, respectively] vs maximal up-regulation by LPS of 447%). However, because it may be a long-lasting effect, it may have physiological significance.

Also, the other monocyte activation marker, ICAM-1, was significantly affected by plasma of pregnant women and women with preeclampsia. After 4 hours of incubation, plasma from pregnant women and plasma from women with preeclampsia even further, significantly increased the percentage of cells expressing ICAM-1 as compared with plasma from nonpregnant women. This plasma-induced increase is comparable with the maximal increase in percentage-positive cells after LPS stimulation, which was also about 13%. This again suggests a strong activating stimulus in the plasma of both pregnant women and women with preeclampsia.

In accordance with the results for ROS production, it may be suggested that factors that up-regulated ICAM-1 in pregnant and preeclamptic plasma differ because not only the percentage ICAM-1–positive cells was increased in women with preeclampsia vs normal pregnant

women, but also this increase in protein expression was accompanied only by increased mRNA expression in women and women with preeclampsia. In accordance with ROS production results, it may be suggested that factors that up-regulated ICAM-1 in pregnant and preeclamptic plasma differ. This is reflected in the fact that the percentage of ICAM-1 positive cells was increased after incubation with preeclamptic plasma versus pregnant plasma, while only after incubation preeclamptic plasma ICAM-1 mRNA was increased as compared with nonpregnant plasma.

The decreased expression of ICAM-1 after 24 hours' incubation with plasma from pregnant patients and patients with preeclampsia was regulated at the transcriptional level because not only ICAM-1 protein expression but also ICAM-1 mRNA was decreased at this time point. The reason for this decreased ICAM-1 expression after 24 hours remains unknown, whereas it is also disputable whether this decrease is of biological significance. It may be speculated, however, that the decreased ICAM-1 expression is due to an overshoot in the mechanisms decreasing monocyte ICAM-1 expression after an activational stimulus.

The present study clearly shows activation of monocytes (ie, a monocytic cell line) after incubation with plasma from both pregnant patients and patients with preeclampsia. What these factors are, or whether the factors in preeclamptic plasma are similar (but in a different concentration) or different from the factors in pregnant plasma, remains unknown. Because poor placentation is a predisposing factor for preeclampsia,[24] it seems likely that these putative factors are produced by the placenta.

Various factors may be suggested to be responsible for the monocyte activation. It has been shown that syncytiotrophoblast membrane fragments (STBMs) are released from the placenta during pregnancy but in increased amounts during preeclampsia.[27] Such STBMs may activate monocytes in both pregnancy and preeclampsia.[27] Other placental debris, such as soluble fetal DNA, which is present during pregnancy and increased

in preeclamptic plasma, also may be involved in monocyte activation.[28]

Other factors involved may be adenosine 5′-triphosphate (ATP) and hemopexin.[29] Hemopexin, an acute-phase reactant, which is associated with vascular permeability and inflammation,[30] is increased in normal pregnancy.[29] ATP, a potent activator of inflammatory cells,[31] is increased during preeclampsia.[29] Also, oxidized lipids,[32] cytokines,[3] and antiangiogenic factors, such as soluble fms-like tyrosine kinase-1 and endoglin, have been implicated in the inflammatory response of preeclampsia.[33]

In summary, the present study showed that in both pregnant and preeclamptic plasma, circulating factors are present, which can activate the monocyte cell line, MM6. Because this monocyte cell line resembles mature monocytes,[21] it seems likely that these factors present in the plasma of both pregnant women and women with preeclampsia also activate peripheral circulating monocytes in these groups of women in vivo. Although the factors remain unknown, in vivo activation of circulating monocytes may result in activation of other cell types, such as endothelial cells. Because we have recently shown that plasma factors do not activate endothelial cells directly,[34] it may be suggested that monocytes, activated by plasma factors, are the initiators of the generalized inflammatory response in pregnancy and preeclampsia. ∎

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