GENERAL OBSTETRICS AND GYNECOLOGY: OBSTETRICS

Altered monocyte function in experimental preeclampsia in the rat

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Objectives: In the present study, we evaluated functional activity of monocytes in experimental preeclampsia induced by low-dose endotoxin infusion.

Study design: Pregnant (n = 12) and cyclic rats (n = 12) were equipped with a permanent jugular vein cannula and infused with either low-dose endotoxin or saline. One day before the infusion, and 4, 24, 72, and 168 hours after the infusion, blood samples (400 µL) were taken and white blood cell (WBC) and differential cell counts were measured. Samples were (re)stimulated with endotoxin, and the percentage of tumor necrosis factor-alpha (TNFα) producing monocytes was measured.

Results: During experimental preeclampsia, monocyte TNFα production is persistently decreased, while total WBC and granulocyte counts are persistently increased compared with normal pregnant rats. No persistent effect of endotoxin was found in cyclic rats.

Conclusion: Because decreased endotoxin-induced TNFα production is a feature of activated monocytes, the present results indicate that monocytes are persistently activated in experimental preeclampsia. Increased WBC counts and granulocyte numbers in these rats also point to an activated inflammatory response.

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Preeclampsia (PE) is the most important and serious complication of pregnancy in the western world.[1] It is defined by the triad of hypertension, proteinuria, and edema, but it may also comprise abnormalities of the liver, the central nervous system, and the blood, as well as disseminated intravascular coagulation.[2] PE is very specific for pregnancy. Although the exact etiology and pathogenesis of this disease remain obscure, there is now consensus that endothelial cells are activated in preeclamptic patients.[3] Recently, it has been shown that circulating inflammatory cells are phenotypically activated in preeclampsia,[4] suggesting an activated inflammatory response (which includes both endothelial cells and inflammatory cells) in preeclampsia.

Using an animal model, we have shown that activation of the inflammatory response in pregnant rats by infusion of low dose endotoxin induced the characteristic signs of preeclampsia, including hypertension and proteinuria.[5] Similar activation of the inflammatory

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response in nonpregnant rats did not induce these preeclampsia-like signs.[5] The difference in reaction to low dose of endotoxin infusion between pregnant and nonpregnant rats can be explained by the fact that the inflammatory reaction induced in pregnant rats is much more intense and persistent compared with nonpregnant rats.[6] This persistent low-dose endotoxin-induced inflammatory reaction in pregnant rats can be observed in the kidneys,[6] but also in circulating inflammatory cells, ie, granulocytes and monocytes.[7] Circulating granulocytes of pregnant endotoxin-treated rats, and not those of nonpregnant endotoxin-treated rats, showed persistent increased expression of CD11a and CD49d and decreased expression of CD62L. Moreover, both monocytes and granulocytes of these pregnant endotoxin-treated rats showed decreased CD14 expression, also an illustration of activation.[7]

The present study was designed to evaluate whether circulating inflammatory cells in experimental preeclampsia are also functionally activated. Monocytes are the first cells to be activated in nonspecific immune responses, and their main function is to produce cytokines such as tumor necrosis factor-alpha (TNFα),[8] by which they activate the inflammatory response. Therefore, we used monocyte TNFα production after an in vitro endotoxin stimulus as a parameter of the function of monocytes and evaluated monocyte TNFα production in experimental preeclampsia in the rat. To this end, pregnant and nonpregnant rats were infused with a low dose of endotoxin or saline (as a control). Blood samples were taken before and at various intervals after infusion, and whole blood was incubated with endotoxin to stimulate cytokine production. Intracellular TNFα production was measured using flow cytometry. Also, we quantified total and differential white blood cell (WBC) numbers.

Material and methods

Experimental animals

We followed the guidelines for the care and use of the animals approved by the local institution.

Female Wistar rats (Harlan; age 3 to 4 months and weighing about 200 g) were kept in a temperature- and light-controlled room (lights on from 6 AM to 6 PM). Daily vaginal smears were taken, and rats with regular 4-day estrus cycles were selected for the experiments. Pregnancy was achieved by housing the female rats on the night of proestrus with a fertile male for 1 night. The next day, when spermatozoa were detected in the smear, was designated as day 0 of pregnancy. On day 0 of pregnancy and on diestrus in cyclic rats, rats were equipped with a permanent jugular vein cannula under fluothane anesthesia, according to the method of Steffens.[9]

Experimental protocol

Pregnant (day 14; n = 12) and cyclic rats (diestrus 2; n = 12) were infused via the permanent jugular vein cannula with either 2 mL endotoxin solution (1.0 μg endotoxin/kg bw [Escherichia coli 0.55:b5, Wittaker MA Bioproducts, Inc, Walkerville, Md]) or 2 mL saline during 1 hour (of each group 6 rats were infused with endotoxin solution and 6 rats were infused with saline). Blood samples (400 μL) were withdrawn from the permanent jugular vein cannula 1 day before the infusion and 4, 24, 72, and 168 hours after the start of the infusion and collected in sterile heparin vacutainer tubes.

Sample handling

Reagents

The following reagents were used: Monensin (Sigma, St Louis, Mo), FACS™ lysing solution (Becton Dickinson Immunocytometry Systems, San Jose, Calif), endotoxin (E coli, 0.55:b5, Wittaker MA Bioproducts, Inc), washing buffer (phosphate-buffered saline [PBS] with 0.5% bovine serum albumin [BSA] and 0.1% NaN_3), saponin (10% in washing buffer), complete RPMI 1640 medium (Gibco BRL, Breda, The Netherlands) supplemented with 60 μg/mL gentamycin, freezing buffer (PBS with 1% BSA and 10% dimethylsulphoxide), and fixation buffer (0.5% paraformaldehyde in PBS).

Sample processing

White blood cells counts

Twenty μL of blood was used to determine total WBC counts. WBC counts were measured with a microcell counter (model Sysmex F800, Toa Medical Electronics Co, Ltd, Kobe, Japan). The percentages of granulocytes, monocytes, and lymphocytes were counted in 3 μL of blood, which was smeared onto a microscope glass and stained with May-Grunnwald-Giemsa according to standard procedures.

Antibodies

Antibodies were purchased from Pharmingen (San Diego, Calif): Cy-Q−labeled mouse antirat CD4 (clone OX-35), fluorescein isothiocynanate (FITC)-labeled mouse antirat CD3 (clone G4.18), phycoerythrin (PE)-labeled hamster antirat TNFα (clone TN-19.12), PE-labeled hamster IgG isotype control (clone G235-2356).

Incubation

Samples were incubated as described previously.[10] In brief, 200 μL of heparinized whole blood was mixed with 200 μL of RPMI and stimulated with endotoxin

Table I Total WBC counts and differential WBC counts in cyclic and pregnant rats (day 14) before infusion of endotoxin or saline

	Cyclic rats		Pregnant rats	
	Total no. of cells(10^9/L)	% of WBC	Total no. of cells(10^9/L)	% of WBC
WBC	7.0 ± 0.2		7.85 ± 0.32*	
Granulocytes	1.3 ± 0.18	18.8 ± 1.97	2.7 ± 0.2*	34.8 ± 2.1*
Lymphocytes	5.5 ± 0.31	78.1 ± 2.17	4.8 ± 0.3*	61.5 ± 2.2*
Monocytes	0.18 ± 0.03	2.61 ± 0.3	0.25 ± 0.04	3.23 ± 0.34
% TNFα producing monocytes	53.7 ± 3.0		65.2 ± 4.3*	

    * Significantly different from cyclic rats ($P < .05$, Mann-Whitney U test).

(2 μg/mL); 200 μL of whole blood was used as the unstimulated control and only mixed with 200 μL of RPMI. In both the stimulated and the unstimulated samples monensin (3 μmol/L) was added. Samples were incubated for 4 hours at 37°C and 5% CO_2.

Sample labeling

Sample labeling was performed as described before.[10] In brief, after incubation, both the stimulated and the unstimulated sample were incubated with 4 μL α-CD4 and 4 μL α-CD3 at saturating dilutions for 30 minutes. After red blood cell lysis WBC were stored at −80°C in freezing buffer until all samples of 1 rat were collected.[10]

When all samples of 1 rat were collected, the samples were collectively thawed and fixed. After permeabilization the samples were incubated α-TNFα. After incubation for 30 minutes, cells were washed with saponin buffer and fixed. They were kept in the dark at 4°C until measurement by flow cytometry within 24 hours.

Flow cytometry

Cells were analyzed with the Coulter Epics Elite flow cytometer (argon-ion 488-nm laser) (Beckman-Coulter, UK). Two thousand monocytes were acquired while life gating on $CD3^-$/$CD4^+$ cells (ie, monocytes). Data were saved for later analysis. Analysis was performed using Winlist 32 (Verity Software House, Inc, Topsham, Me).

Data analysis

Data were analyzed as described before.[10] A single parameter histogram was defined for the $CD3^-$/$CD4^+$ monocytes to evaluate the percentage of monocytes producing intracellular TNFα: using the unstimulated control sample, a linear gate was set so that 99% of the unstimulated cells were negative for TNFα. This gate was copied to the histogram of the stimulated cells to evaluate the percentage of TNFα producing monocytes after endotoxin stimulation.

Statistics

Results are expressed as mean ± standard error of mean (SEM). Because the present study was a longitudinal

study in which rats were tested before and after the infusion of either endotoxin or saline, paired testing was used. Therefore, post-infusion WBC counts, differential cell counts, and percentage TNFα producing monocytes were tested vs preinfusion values using Wilcoxon signed rank. Moreover, differences in preinfusion values between pregnant and cyclic rats were tested using Mann-Whitney U test. Differences were considered significant if $P < .05$.

Results

Experimental animals

After infusion of either saline or endotoxin and blood sampling, at the end of the experiments, pregnant rats were allowed to deliver. Rats that did not deliver between 5 PM on day 21 and 5 PM on day 22 were discarded from the experiments. All pups were weighed 1 day after delivery. The number of pups, as well as the pup weight, did not differ between saline- and endotoxin-infused rats (mean number of pups 9.8 ± 1.6 and 7.5 ± 2.1 for saline and endotoxin infused rats, respectively; mean pup weight: 7.68 ± 0.4 and 7.59 ± 0.87 for saline- and endotoxin-infused rats, respectively).

Total WBC counts

Total WBC counts were significantly increased in day 14 pregnant rats compared with cyclic rats (Table I). As can be seen from Figure 1, endotoxin infusion significantly decreased total WBC counts at 4 hours after the infusion. Thereafter, WBC counts returned to normal, and were significantly increased at 72 hours after the infusion. In cyclic endotoxin-treated rats, WBC counts then returned to normal, while in pregnant endotoxin-treated rats, ie, rats with experimental preeclampsia, WBC counts remained increased until the end of pregnancy, until 168 hours after the infusion.

Differential WBC counts

In the preinfusion sample, total lymphocyte count was decreased, while total granulocyte count was increased in pregnant rats compared with cyclic (Table I).
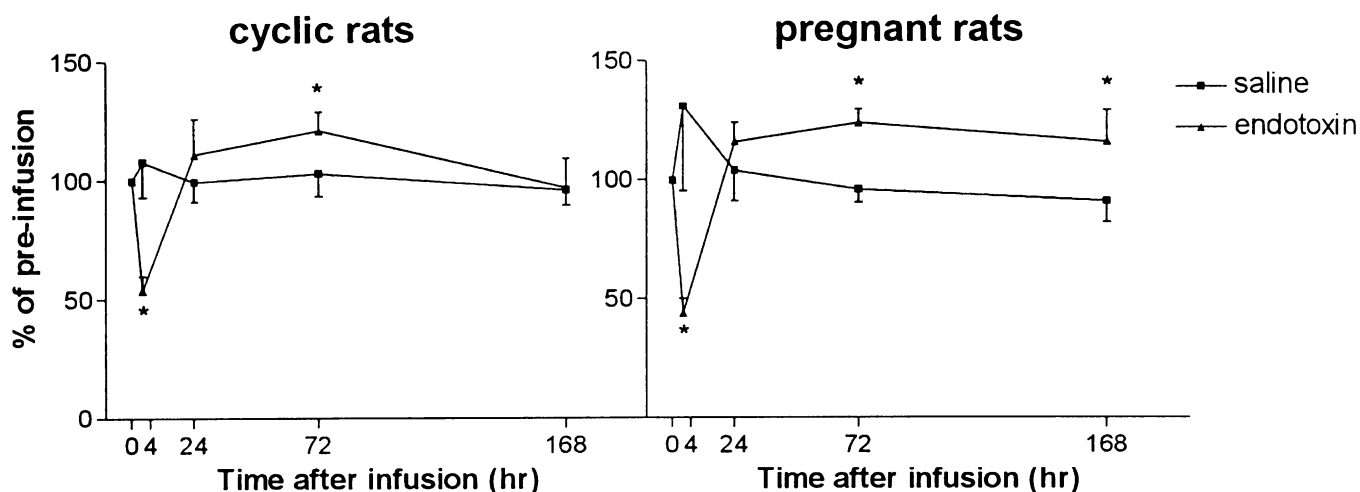
cyclic rats   ## pregnant rats

Figure 1   Total white blood cell (WBC) counts in cyclic (left panel) and pregnant (right panel) rats at various intervals after the infusion of endotoxin (triangles) or saline (squares). Results are expressed as percentage of the preinfusion value. *Significantly different from preinfusion value ($P < .05$, Wilcoxon).

After endotoxin infusion in cyclic rats, granulocyte numbers were increased at 4 and 24 hours after the start of the infusion, and were not significantly different from preinfusion values at 72 and 168 hours after the infusion (Figure 2). Endotoxin infusion in pregnant rats did not affect granulocyte numbers at 4 hours after the endotoxin infusion. However, granulocyte numbers were significantly increased at 24 hours after the start of the infusion and remained significantly elevated in rats with experimental preeclampsia until the end of pregnancy.

Endotoxin infusion also affected monocyte and lymphocyte numbers, but only at 4 hours after the infusion, at which time both monocyte number and lymphocyte number were significantly decreased. No difference between pregnant and cyclic rats could be observed (Figure 2).

Monocyte TNFα production after a second in vitro endotoxin challenge

The preinfusion percentage of TNFα producing monocytes after ex vivo activation with endotoxin was significantly increased on day 14 of pregnancy compared with cyclic rats (Table I). In both pregnant and nonpregnant rats, infusion of saline did not affect the percentage of monocytes producing TNFα after the in vitro endotoxin challenge (Figure 3).

In cyclic rats, infusion of endotoxin significantly decreased the percentage of monocytes producing TNF-α after a second endotoxin challenge at 4 and 24 hours after the infusion. In pregnant rats, the percentage of monocytes producing TNFα after a second endotoxin stimulus was also significantly decreased at 4 and 24 hours after the endotoxin infusion, but then remained decreased until the end of pregnancy, which was 7 days after the infusion.

Comment

In this study we evaluated monocyte function and peripheral total and differential WBC counts in experimental preeclampsia. Experimental preeclampsia was induced by low-dose endotoxin infusion, as described before.[5] We found that WBC counts were persistently increased in rats with experimental preeclampsia, which is caused by persistently increased numbers of granulocytes. Moreover, we found that monocytes from rats with experimental preeclampsia showed a persistent decreased production of TNFα after restimulation with endotoxin in vitro.

In the present study, in nonpregnant endotoxin-treated rats, we observed a decreased TNFα production after restimulation with endotoxin, indicating that these cells are "endotoxin tolerant." Endotoxin tolerance is a well-known phenomenon of activated monocytes, and is characterized by decreased production of, for instance, TNFα, after a second endotoxin stimulus.[11] Endotoxin tolerance can be observed in peripheral monocytes isolated from septic patients or from patients after surgery.[12,13] In cyclic rats, the endotoxin tolerance is most obvious immediately (4 hours) and at 24 hours after the infusion of endotoxin, and thereafter, disappears. This is in line with studies by others,[14] who also showed a prominent endotoxin tolerance after rechallenge with endotoxin shortly after the primary stimulation, and lasting at least until 24 hours after the first endotoxin challenge.

Interestingly, in "pregnant" monocytes, endotoxin infusion induced a much more sustained endotoxin tolerance compared with nonpregnant monocytes, indicating that in experimental preeclampsia, monocyte function has persistently changed. Because endotoxin tolerance seems to be a feature of activated monocytes,[11]
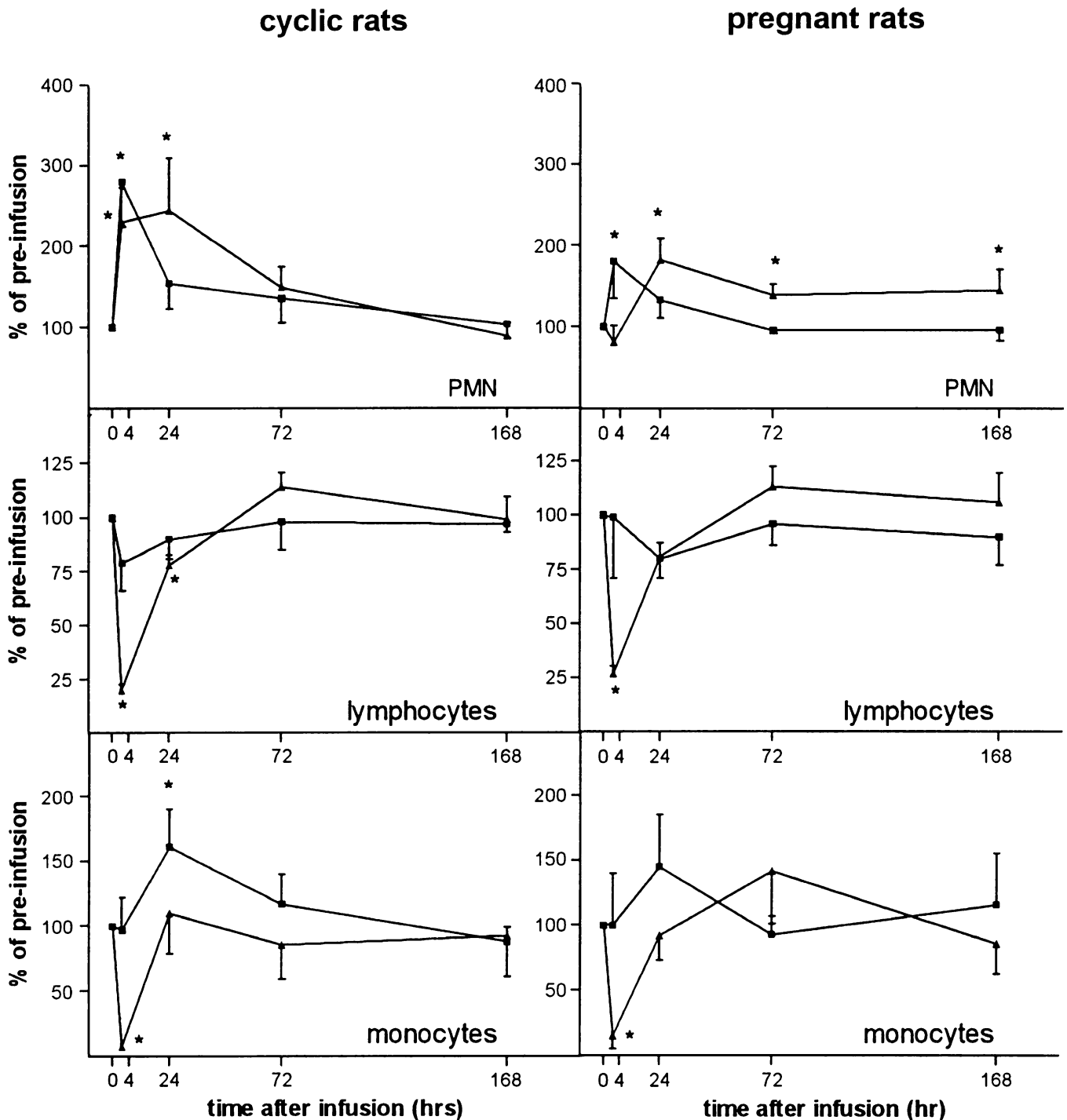
cyclic rats   pregnant rats

Figure 2 Differential white blood cell counts in cyclic (left panels) and pregnant (right panels) rats at various intervals after the infusion of endotoxin (triangles) or saline (squares). Top panels represent granulocyte numbers; middle panels represent lymphocytes; bottom panels represent monocytes. Results are expressed as a percentage of the preinfusion value. *Significantly different from the preinfusion value ($P < .05$, Wilcoxon).

the present results corroborate our previous study in that during experimental preeclampsia, monocyte CD14 expression was persistently decreased, indicating that monocytes were persistently activated, while in non-pregnant rats, monocytes are only transiently activated.[7] Although it is well known that endotoxin tolerance is

a feature of activated monocytes, the mechanism by which it is induced still remains largely unknown.[7] Various putative mechanisms may account for the endotoxin tolerance in cyclic rats in the present study, such as down-regulation of monocyte TLR4,[15] impaired activation of the transcription factor nuclear factor-
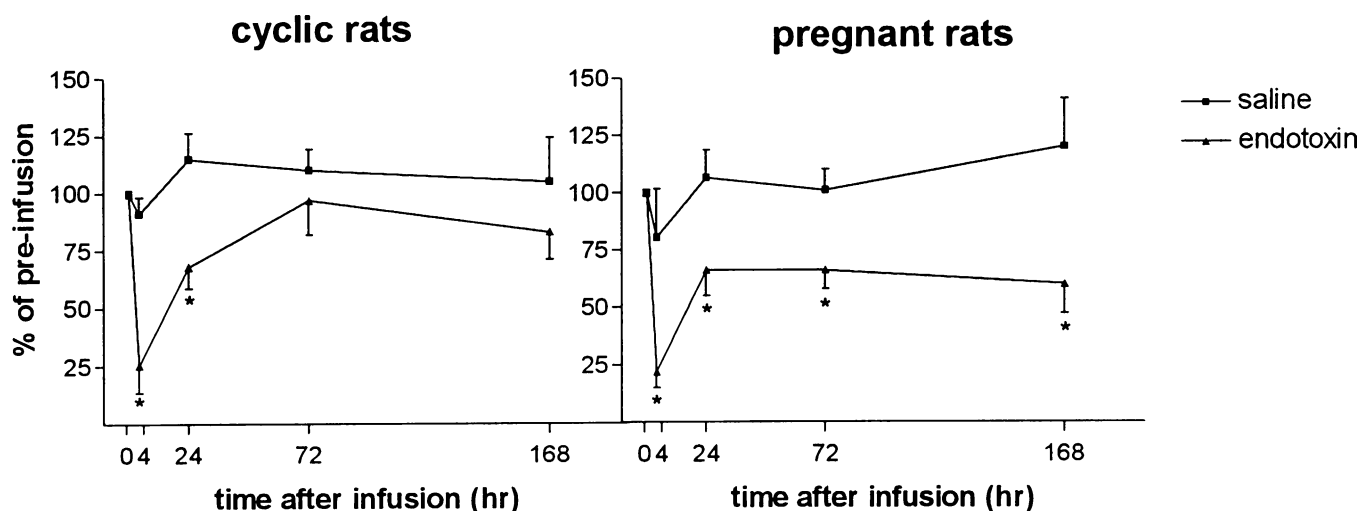
Figure 3 Percentage of monocytes producing TNFα after endotoxin (triangles) or saline (squares) infusion. Left panel shows monocytes of cyclic rats, and the right panel shows monocytes of pregnant rats. Results are expressed as a percentage of the preinfusion value. *Significantly different from the preinfusion value ($P < .05$, Wilcoxon).

kappa B (NF-κB),[16] or attenuation of phosphorylation of p38 MAP kinase.[17] Whether similar mechanisms may account for the endotoxin tolerance in pregnant monocytes immediately after the infusion remains to be investigated.

Prolonged endotoxin-tolerance in pregnant rats only may be caused by a difference in leukocyte cytokine production between pregnant and nonpregnant individuals. Interferon-γ (IFN-γ) and granulocyte macrophage colony-stimulating factor (GM-CFS) are known to reverse or prevent endotoxin tolerance.[18] In vivo in nonpregnant rats, it may be possible that IFN-γ, which is produced by natural killer (NK) cells after endotoxin treatment,[19] may help restore endotoxin tolerance in monocytes. Interestingly, we have previously shown that, at least in human pregnancy, NK cell IFN-γ production is significantly decreased in pregnant women compared with nonpregnant women.[20] Another mechanism for the prolonged monocyte activation may be delayed apoptosis. Although we are not aware of any publications about monocyte apoptosis during pregnancy, it has been shown that granulocyte apoptosis is delayed during human pregnancy.[21] It is feasible that during pregnancy, monocyte apoptosis is also delayed, and therefore, activated monocytes remain in the circulation so that endotoxin tolerance and the inflammatory response can persist more pronounced than in nonpregnant rats.

In the present study, we also measured total WBC counts and differential blood cell counts in rats with experimental preeclampsia. WBC counts of pregnant rats (preinfusion value) were significantly increased compared with nonpregnant because of increased numbers of granulocytes in these pregnant rats. In both pregnant and cyclic rats, after infusion of endotoxin, WBC counts

first decreased, followed by a return to normal values, and even an increase of WBC counts at the later intervals. The decrease in WBC counts immediately after the infusion is caused by a decrease in the numbers of monocytes and lymphocytes, which is similar in pregnant and cyclic rats. This decrease may be a result of extravasation or margination of these cells. At 24 hours after the infusion, monocyte and lymphocyte numbers returned to normal values as a result of increased production and/or demargination. Only in pregnant endotoxin-treated rats, ie, rats with experimental preeclampsia, did total WBC numbers remain increased until 7 days after the infusion (which is the end of pregnancy). This is caused by persistent increased numbers of granulocytes. These data are in line with clinical observation in preeclampsia: both WBC counts and granulocyte counts are increased in preeclampsia.[22,23]

The present results show that the most obvious differences between pregnant and cyclic endotoxin-infused rats were observed in the later intervals. Only granulocyte numbers immediately after endotoxin infusion differed between pregnant and nonpregnant rats. In nonpregnant rats, endotoxin infusion increased the number of circulating granulocytes, only at 4 hours. This phenomenon is also most likely the consequence of marginating granulocytes being released into the circulation after endotoxin infusion. In contrast to nonpregnant rats, in pregnant rats, granulocyte numbers did not increase 4 hours after the endotoxin infusion. This may be because of an increased efflux of these cells from the circulation caused by infiltration into various tissues, for instance, the glomeruli of the kidneys.[6] At the later intervals, despite the fact that increased infiltration of granulocytes into the glomeruli of the kidneys can still be observed,[6] circulating granulocyte numbers are

increased in pregnant endotoxin-treated rats. This may illustrate a decreased apoptosis of granulocytes in pregnancy,[21] or increased production of granulocytes after activation of the inflammatory response during pregnancy.

In summary, the present study showed that in experimental preeclampsia in the rat, which is induced by endotoxin infusion, monocyte TNFα production upon a second endotoxin stimulus is persistently inhibited. Monocytes are in a so-called state of endotoxin-tolerance. Because this endotoxin tolerance is a feature of activated monocytes, these results suggest that in experimental preeclampsia, monocytes are activated. Along with this persistent activation of monocytes during experimental preeclampsia, we found persistent increased number of WBC and granulocytes. These results are consistent with results in human preeclampsia[4] and with our hypothesis of preeclampsia being the result of an inflammatory response.[24] The present study was not set up to measure TNFα production of circulating monocytes. It may, however, be suggested that the activated circulating monocytes in experimental preeclampsia produce TNFα. This may be in line with the human situation, in which increased levels of TNFα have been found in the maternal circulation of women with preeclampsia.[25] Because monocytes from women with preeclampsia are phenotypically activated, it may be suggested that these monocytes are also endotoxin tolerant. At present, we are studying possible endotoxin tolerance in monocytes of women with preeclampsia.

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