

Endotoxin-induced cytokine production of monocytes of third-trimester pregnant women compared with women in the follicular phase of the menstrual cycle

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OBJECTIVE: Little is known about the function of the innate immune response during pregnancy. We therefore investigated monocyte cytokine production, as a measure of monocyte function, in pregnant women compared with nonpregnant women.

STUDY DESIGN: Whole blood of women in the follicular phase (day 5-6) and of healthy pregnant women (30 weeks) was collected and stimulated with endotoxin (2 µg/mL). After incubation for 4 hours (37°C, 5% carbon dioxide), red blood cells were lysed and white blood cells were permeabilized, followed by staining with anti-CD14 (fluorescein isothiocyanate labeled) and with phycoerythrin-labeled tumor necrosis factor- α , interleukin-1 β , or interleukin-12. The cells were analyzed by flow cytometry after fixation. Results are expressed as a percentage cytokine producing cells after endotoxin stimulation. Statistical analysis was performed with the Mann-Whitney *U* test ($P < .05$).

RESULTS: Compared with the percentage endotoxin-induced cytokine producing peripheral monocytes in women in the follicular phase, this percentage in pregnancy was decreased for interleukin-12 (mean 6.63 ± 1.34 vs 3.34 ± 0.87 , $P < .05$) and tumor necrosis factor- α (mean 50.20 ± 5.80 vs 31.29 ± 5.57 , $P > .05$). No significant difference was seen in the production of interleukin-1 β (mean 58.22 ± 11.09 vs 47.18 ± 7.88 , $P > .05$).

CONCLUSION: The percentage of interleukin-12 and tumor necrosis factor- α producing monocytes is decreased in pregnant women compared with nonpregnant women, suggesting that pregnancy is a proinflammatory state. (Am J Obstet Gynecol 2003;188:1073-7.)

Key words: Cytokine production, interleukin-12, tumor necrosis factor- α , monocytes, pregnancy, interleukin-1 β , endotoxin

Pregnancy is an immunologic paradox: the semiallogeneic blastocyst is not rejected by the immune system of the mother.¹ To solve this paradigm, research has focused on the specific immune response during pregnancy. It has been suggested that type 1 cytokines are harmful for maintenance of pregnancy, whereas type 2 cytokines appear to be protective for the fetus.²⁻⁵ Therefore, during pregnancy the immune response is shifted from a T_H1

type response (cellular immune response) to a T_H2 type immune response (humoral immune response).⁶ Indeed, we have shown that lymphocytes from pregnant women show a decreased production of T_H1 cytokines compared with nonpregnant women. This results in a decreased T_H1/T_H2 ratio during pregnancy.⁷

Little is known about the innate immunity (ie, inflammatory responses) during pregnancy. Previous research from our laboratory has shown that endotoxin-induced inflammatory responses are much more intense and persistent in pregnant rats compared with nonpregnant rats.⁸ This suggests that pregnancy is a proinflammatory condition, which is in line with other data demonstrating that circulating monocytes and granulocytes are activated during pregnancy in humans⁹ and in rats.¹⁰ Sacks et al¹¹ hypothesized that particulate or soluble placental products may have modulating effects on the maternal immune response during human pregnancy. Monocytes could possibly be target cells and could be activated.¹¹

Monocytes are the first cells to be activated in nonspecific immune responses. On activation (eg, by endo-

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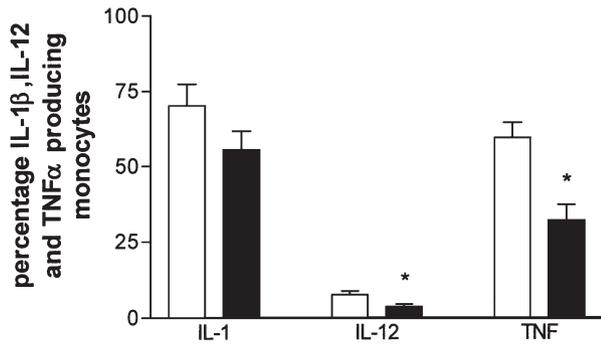


Figure. Percentage of cytokine producing monocytes in follicular phase (open bars) and third trimester pregnancy (closed bars). Asterisk, Statistically significant decreases (Wilcoxon, $P < .05$).

toxin), they produce various cytokines such as tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β , and IL-12.¹² Because monocyte cytokine production can vary with the state of activation of monocytes,¹³ we used cytokine production of monocytes after in vitro stimulation with endotoxin (lipopolysaccharide [LPS]) as a parameter for the state of activity of monocytes. Thus, endotoxin-induced monocyte cytokine production of pregnant women in the third trimester was compared with monocyte cytokine production of women in the follicular phase. Women in the follicular phase were chosen because we previously demonstrated a difference in monocyte cytokine production in the follicular phase compared with the luteal phase.¹⁴ The intracellular productive capacity of TNF- α , IL-1 β , and IL-12 of the peripheral blood monocytes was measured by flow cytometry.

Material and methods

Reagents for cell activation and cell staining. The following is a list of reagents used: monensin (Sigma, St Louis, Mo), FACS lysing solution (Fluorescence Activated Cell Sorting, Becton Dickinson, San Jose, Calif), FACS permeabilizing solution (Becton Dickinson), complete Roswell Park Memorial Institute (RPMI) 1640 (GIBCO BRL, Breda, The Netherlands) medium supplemented with 60 $\mu\text{g}/\text{mL}$ gentamicin, washing buffer (phosphate-buffered saline solution [PBS] with 0.5% bovine serum albumin and 0.1% sodium azide), and fixation buffer (0.5% paraformaldehyde in PBS).

Subjects. After institutional approval of the local ethics committee was obtained and after the women had signed informed consent, blood samples were taken from healthy nulliparous women (aged 18-35 years, $n = 17$) at 30 weeks' amenorrhea (range 29-31 weeks, recruited from our "outpatient clinic for midwives") and from healthy nonpregnant women (aged 18-35 years, $n = 15$) with a regular menstrual cycle length between 26 and 32 days in their follicular phase (days 6-9 of the cycle) (recruited from hospital staff and students). For both

groups, exclusion criteria were evidence of treatment with antibiotics or flu-like symptoms within 14 days of the blood sampling as well as the presence of any known diseases. Blood samples (20 mL) were obtained in two Vacutainer blood-collecting tubes (Becton Dickinson, Rutherford, NJ); one tube contained sodium heparin and was used to evaluate intracellular cytokine production, and the other contained EDTA and was used for white blood cell (WBC) counts (using a micro cell counter, model Sysmex F800, Toa Medical Electronics Co, Ltd, Kobe, Japan).

Sample processing

Antibodies. The following monoclonal antibodies were used, unless stated otherwise: fluorescein isothiocyanate (FITC)-labeled mouse antihuman CD14 (clone UCHM1; IQ Products, Groningen, The Netherlands); phycoerythrin (PE)-labeled mouse anti-human TNF- α (clone Mab11; BD Biosciences Pharmingen, San Diego, Calif), PE-labeled mouse anti-human IL-12 (clone C11.5; Pharmingen), PE-labeled mouse anti-human IL-1 β (clone AS10; Becton Dickinson).

Incubation. Immediately after sampling, 3 mL of heparinized whole blood was mixed with 3 mL of RPMI and stimulated with LPS (2 $\mu\text{g}/\text{mL}$; 055.B5 *Escherichia coli*, Bio Whittaker, Walkersville, Md) for 4 hours at 37°C and 5% carbon dioxide. Three milliliters of heparinized whole blood was used as unstimulated control and only mixed with 3 mL of RPMI. In both the stimulated and unstimulated sample, monensin (3 $\mu\text{mol}/\text{L}$) was added to enable accumulation of the cytokines in the Golgi complex by interrupting the intracellular processes.

Sample labeling. After stimulation, both samples were aliquoted (0.2 mL per tube) and incubated with anti-CD14 (5 μL) (for identifying monocytes) for 30 minutes. After that, 1 mL of lysing buffer was added to all tubes for lysing of red blood cells. After 5 minutes' incubation at room temperature in the dark, all tubes were centrifuged and aspirated. The remaining pellets were resuspended in 0.5 mL of permeabilization buffer and incubated at room temperature (RT) in the dark for 10 minutes. Then cells were washed with ice-cold washing buffer once. After centrifugation and aspiration, stimulated and nonstimulated aliquots were incubated at RT in the dark for 30 minutes with either 5 μL of anti-TNF- α , anti-IL-1 β , anti-IL-12, or isotype control IgG₁ at saturating dilutions. After washing with the washing buffer, cells were fixed with ice-cold 0.5% paraformaldehyde in PBS and kept at 4°C in the dark until measured (within 24 hours).

Flow cytometry. Cells were analyzed with the Coulter Epics Elite flow cytometer (argon ion 488 nm laser) (Coulter, Miami, Fla). Two thousand monocytes were acquired while life gating on monocytes with the use of CD14⁺ cell signal and saved for later analysis. Analysis was performed with Winlist32 (Verity Software House, Topsham, Me).

Table. Total WBC count and differential counts in the follicular phase of the ovarian cycle and the third trimester pregnancy

Variable	Follicular phase of menstrual cycle		Third-trimester pregnancy	
	Total No. of cells (10 ⁷ /L)	Percentage of WBC	Total No. of cells (10 ⁷ /L)	Percentage of WBC
WBC	550 ± 26		1151 ± 72*	
Granulocytes	336 ± 19	61.1 ± 2.9	860 ± 65*	74.7 ± 1.2*
Monocytes	23.1 ± 3	4.2 ± 0.7	45 ± 5*	3.9 ± 0.4
Lymphocytes	181 ± 19	33 ± 2.6	158 ± 14	13.7 ± 1.1*

Values are mean ± SEM.

*Statistically significant (Wilcoxon, $P < .05$).

Intracellular cytokines. During analysis, a gate was set on monocytes using α CD-14 signal. Single parameter fluorescence histograms were defined for evaluation of intracellular cytokine production. During analysis, it was checked that the isotype control completely overlapped the unstimulated sample, indicating that monocytes in the unstimulated sample did not produce cytokines. This was the case in all samples and for all cytokines. With the use of the unstimulated control sample, linear gates were set in the histograms so that at least 99% of the unstimulated cells were negative for cytokine production. This gate was then copied to the stimulated sample. Results are expressed as percentage positive cells in the stimulated blood sample.

Differential blood cell counts. With the use of forward and side scatter characteristics, a gate was set on the total leukocyte population.

By use of forward and side scatter characteristics as well as α CD14 signal, lymphocyte, monocyte, and granulocyte populations were defined in an unstimulated aliquote of each sample and percentage lymphocytes, monocytes, and granulocytes of the total leukocyte population were evaluated.

Statistics. Results are expressed as mean ± SEM. To evaluate differences between the follicular phase and pregnancy, the Mann-Whitney U test was used. Differences were considered to be significant if $P < .05$.

Results

The Table shows the mean total leukocyte counts in the follicular phase of the menstrual cycle and during third-trimester pregnancy as well as the percentage of monocytes, granulocytes, and lymphocytes. There was a significant increase in total number of white blood cells, monocytes, and granulocytes during pregnancy compared with the follicular phase.

The Figure shows the percentage of cytokine-producing monocytes after stimulation with LPS in pregnant women compared with women in the follicular phase. As can be seen in this illustration, the percentage of IL-12- and TNF- α -producing monocytes was significantly

decreased in pregnant women compared with women in the follicular phase of the menstrual cycle (Mann-Whitney U test, $P < .05$).

No significant difference was seen in IL-1 β -producing monocytes between the groups.

Comment

It is well known that specific immune responses differ between pregnant women and nonpregnant women: lymphocytes show a decreased production of type 1 cytokines, interferon gamma, and IL-2 in pregnant women compared with nonpregnant women.⁷ The current study shows that also monocyte cytokine production, after in vitro stimulation with endotoxin, differed between pregnant and nonpregnant women. In our study the percentage of IL-12- and TNF- α -producing peripheral monocytes in women in the third trimester of pregnancy after in vitro stimulation with endotoxin was significantly decreased compared with women in the follicular phase of the menstrual cycle.

To our knowledge, this study is the first study to evaluate cytokine production in monocytes after stimulation in vitro of whole blood of pregnant women and whole blood of nonpregnant women. We measured intracellular production of TNF- α , IL-1 β , and IL-12 using flow cytometry. This enabled us to detect cytokine production at the single cell level. TNF- α and IL-1 β were chosen for their central role in the induction of the LPS effect¹⁵; IL-12 was chosen for its role in providing a link between innate immunity and adaptive immunity. IL-12 produced by monocytes is a powerful factor for generation of a T_H1 type immune response.¹⁶

Endotoxin (LPS), a component of the outer cell membrane of gram-negative bacteria, is a powerful stimulator of monocytes. When endotoxin is introduced in vitro, monocytes produce cytokines like TNF- α , IL-1 β , and IL-12.¹⁴ When introduced in vivo, endotoxin initiates a cascade of inflammatory pathways in healthy humans but also induces a temporary state known as LPS tolerance.^{17,18} LPS tolerance, which can be demonstrated in activated monocytes, is characterized by decreased pro-

duction of TNF- α , IL-1 β , and IL-12 after an endotoxin stimulus.¹³ This LPS tolerance can be observed in peripheral monocytes isolated from septic patients or from patients after surgery.^{13,19,20} Therefore, LPS tolerance appears to be an adaptive host immune response, which is not specific for prior exposure to endotoxin.

Decreased production of monocyte TNF- α and IL-12 during pregnancy appears at first to contradict with our hypothesis that pregnancy is a proinflammatory condition. However, as stated before, activated monocytes produce less cytokines (cf. sepsis and surgery). We therefore hypothesize that decreased production of TNF- α and IL-12 is the result of a state of LPS tolerance because monocytes are activated during pregnancy. This theory is in line with the fact that during pregnancy the number of monocytes and granulocytes is increased, also suggesting a proinflammatory situation. Moreover, monocyte surface expression of the endotoxin receptor CD14 is increased during pregnancy,²¹ indicating that monocytes are active. These results may be also consistent with the observation that pregnant animals are highly susceptible for the generalized Schwartzman reaction,²² a reaction that is mediated by monocytes.

The question arises what factors are responsible for modulating cytokine production of monocytes during pregnancy. One possibility is that increased progesterone during pregnancy is responsible for the fact that production of TNF- α and IL-12 is decreased during pregnancy. However, Polan et al²³ demonstrated that endotoxin-stimulated peripheral blood monocyte levels of both TNF- α messenger RNA (mRNA) and IL-1 β mRNA increased, when incubated with physiologic levels of progesterone and 17 β -estradiol concentrations.^{23,24} Moreover, we demonstrated that monocyte intracellular production of TNF- α and IL-1 β after in vitro LPS is increased in parallel with increased progesterone and estrogens during the luteal phase compared with the follicular phase.¹⁴ Therefore, if hormones such as progesterone and estradiol have an effect on monocyte cytokine production, they increase monocyte cytokine production. The current decrease of TNF- α and IL-12 is most likely not due to progesterone and estradiol.

Other factors responsible for the modulation of monocytes in pregnancy can be found in or from the placenta. This is in line with the suggestion of Redman et al,²⁵ who suggested that syncytiotrophoblast microfragments (STBM) in the maternal circulation are a trigger to stimulate the inflammatory response in pregnancy. It is suggested that in normal pregnancy, fetal cells, cellular particles, cytoplasmic proteins, and soluble fetal DNA are shed from the STB surface into the maternal blood, as a part of the normal turnover of the syncytium. These fragments can directly or indirectly influence monocytes. Indeed, preliminary studies from our laboratory showed a decreased production of TNF α and IL-12 in monocytes

from women in the luteal phase when stimulated with endotoxin in the presence of STBM (manuscript in preparation).

The current results are in line with previous results from our laboratory showing that during pregnancy the immune response is shifted toward a T_H2 response⁷: in the current study we found a decreased production of type 1 cytokines by monocytes during pregnancy. Especially the decreased production of IL-12 is interesting because IL-12 is a powerful inducer of T_H1 responses. Decreased production of IL-12 in our study, however, contradicts to the results of Sacks et al⁹, who found increased production of IL-12 by monocytes during pregnancy compared with nonpregnant individuals. They, however, used isolated peripheral blood mononuclear cells and stimulated them with both LPS and IFN- γ .⁹ Apparently, either the isolation procedure or the different stimulation method affects IL-12 production.

In conclusion, we observed decreased percentages of monocytes producing TNF- α and IL-12 after endotoxin stimulation during pregnancy and speculate that this may be because monocytes are activated as a result of the condition of pregnancy; they are in a state of "LPS-tolerance." These results are in line with the hypothesis that pregnancy is a proinflammatory condition.

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