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## Total white blood cell counts and LPS-induced TNF $\alpha$ production by monocytes of pregnant, pseudopregnant and cyclic rats

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### Abstract

Pregnancy in the rat may be associated with an activated innate immune system. Therefore, we investigated monocyte function as well as total white blood cell (WBC) counts during the follicular phase of the ovarian cycle, pregnancy and pseudopregnancy in the rat. Rats were equipped with a permanent jugular vein cannula, and 0.43 ml blood samples were taken from this cannula during the 4 days of the regular oestrus cycle of the rat ( $n = 12$ ). Thereafter, six rats were rendered pregnant, and the other six rats were rendered pseudopregnant according to standard methods. Blood samples were withdrawn from the cannula on days 4, 7 and 11 of pseudopregnancy and on days 4, 7, 11 and 20 of pregnancy. From each blood sample, 0.4 ml was stimulated with lipopolysaccharide (LPS) and monocyte intracellular cytokine production measured using flow cytometry. 30  $\mu$ l of the blood was used to measure WBC counts and differential WBC counts. The results showed that the number of WBC was significantly

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increased only on day 11 of pregnancy compared with the follicular phase, and that this was due to the increased numbers of polymorphonuclear (PMN) cells. The percentage of TNF $\alpha$ -producing monocytes was increased on all days of pseudopregnancy and on day 11 of pregnancy. The fact that the percentage of monocytes producing TNF $\alpha$  upon an LPS stimulus was increased during the post-implantation phase of pregnancy and during pseudopregnancy as compared to the follicular phase may indicate that these conditions are proinflammatory conditions. For the post-implantation phase of pregnancy, this is once more stressed by the increased numbers of WBC and PMN.

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**Keywords:** Monocytes; TNF $\alpha$ ; White blood cells; Progesterone; Placenta; Pregnancy

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## 1. Introduction

Pregnancy is associated with changes in the immune response which are necessary for the semiallogeneic blastocyst to be able to implant. Most research has focussed on lymphocyte cytokine production and we have previously shown that during pregnancy, the peripheral-specific immune response is shifted away from a type 1 (i.e. cellular) immune response towards a type 2 (i.e. humoral) immune response (Veenstra van Nieuwenhoven et al., 2002). Others have, however, suggested that this may be an oversimplification (Chaouat et al., 2002). It has also been suggested that to maintain resistance to infection, the activity of the maternal innate immune system, represented by monocytes and granulocytes, is increased (Sacks et al., 1999). This is not only apparent from the increased numbers of circulating monocytes and granulocytes (Letsky, 1980; Veenstra van Nieuwenhoven et al., 2002), but also from the fact that circulating monocytes and granulocytes show an activated phenotype (Sacks et al., 1998). Moreover, others have shown increased monocyte phagocytosis and respiratory burst activity (Shibuya et al., 1987) or granulocyte activation (Shibuya et al., 1987; Sacks et al., 1998) during human pregnancy.

Although there is clear evidence of systemic activation of the innate immune system in human pregnancy, relatively little is known about other species. The fact, however, that pregnant experimental animals of various species have increased sensitivity to lipopolysaccharide (LPS, a potent activator of innate immune responses, especially monocytes) compared to non-pregnant animals (Beller et al., 1985), suggests that, also during pregnancy in other species, the innate immune response has changed. Indeed, in pregnant rats, infusion of a very low dose of LPS induced a generalised inflammatory response, which is much more intense and persistent compared with non-pregnant rats (Faas et al., 1995). Moreover, in line with human experiments, we observed recently that during rat pregnancy both monocytes

and granulocytes show an activated phenotype (Faas et al., 2000). These data suggest that, in line with human pregnancy, the innate immune system is also activated during pregnancy in the rat. More direct data about monocyte or granulocyte function during pregnancy in the rat, however, are lacking.

Therefore, the present study was set up to evaluate monocyte function in rat pregnancy and compare it with monocyte function during the follicular phase and pseudopregnancy in the rat. Blood samples were taken from rats in the follicular phase, pseudopregnancy and pregnancy, and whole blood was stimulated with LPS in vitro after which intracellular production of TNF $\alpha$  was measured as a parameter of monocyte function. Since increased white blood cell (WBC) counts and increased numbers of granulocytes and monocytes are also indicators for a proinflammatory situation, total and differential blood cell counts were also measured.

## 2. Materials and methods

### 2.1. Experimental animals

All experiments were conducted in accordance with the Guide for the Care and Use of Agriculture Animals in Agriculture Research and Teaching (1988). Female Wistar rats (Harlan; age 3–4 months and weighing about 200 g) were kept in a temperature- and light-controlled room (lights on from 6 A.M. to 6 P.M.). Daily vaginal smears were taken and rats with regular 4-day oestrus cycles (i.e. rats in the follicular phase of the ovarian cycle; cyclic rats) were selected for the experiments. Twelve regularly cycling rats were equipped with a permanent jugular vein cannula under fluothane anaesthesia, according to the method of Steffens (1969). At the autopsy, at the end of the experiments, the cannulae were checked for signs of infection. No signs of infection were found in any of the rats used in the experiments.

Pregnancy was achieved by housing the female rats on the night of pro-oestrus with a fertile male for one night. The next day, when spermatozoa were detected in the smear, was designated as day 0 of pregnancy. As rats do not exhibit a spontaneous luteal phase (Freeman, 1988), a luteal phase (pseudopregnancy) was induced by electrical stimulation of the cervix uteri on pro-oestrus (at 17:00 h) and on oestrus (at 15:00 h) according to standard methods. Plasma progesterone concentrations were measured on di-oestrus in rats in the follicular phase and on day 10 of pseudopregnancy (radioimmunoassay, according to the method of Jong et al. (1974)). Mean plasma concentration of progesterone was  $40.1 \pm 2.23$  nM during the follicular phase

and significantly increased to  $110.2 \pm 6.5$  nM on day 10 of pseudopregnancy, indicating that pseudopregnancy was achieved.

### 2.1.1. Experimental protocol

Blood samples (430 µl) were withdrawn from the permanent jugular vein cannula of the cyclic rat at each day of the 4-day oestrus cycle and collected in sterile heparin vacutainer tubes. To avoid withdrawing too much blood in a short period of time, blood was withdrawn from 2 days in one cycle and from the other 2 days in the next cycle.

After blood had been taken from all 4 days of the oestrus cycle, six of the rats were rendered pregnant, and the other six were rendered pseudopregnant. 430 µl blood samples were taken on days 4, 7, 11 and 20 of pregnancy and on days 4, 7 and 11 of pseudopregnancy.

## 2.2. Sample handling

### 2.2.1. Reagents

The following reagents were used: monensin (Sigma, St. Louis, MO), FACS™ lysing solution (Becton Dickinson Immunocytometry Systems, San Jose, CA), LPS (*E. coli*, 0.55:b5, Witten MA Bioproducts, Inc., Walkerville, MD), washing buffer (phosphate-buffered saline with 0.5% bovine serum albumin and 0.1% NaN<sub>3</sub>), saponin (10% in washing buffer), complete RPMI 1640 medium (GIBCO BRL, Breda, The Netherlands) supplemented with 60 µg/ml gentamycin, freezing buffer (phosphate-buffered saline with 1% bovine serum albumin and 10% dimethylsulphoxide) and fixation buffer (0.5% paraformaldehyde in PBS).

### 2.2.2. Sample processing

2.2.2.1. *White blood cell counts.* 20 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). 3 µl of blood was smeared onto a microscope glass and after staining with May-Grunnwald-Giemsa according to standard procedures, percentages of granulocytes, monocytes and lymphocytes were counted.

2.2.2.2. *Antibodies.* Antibodies were purchased from Pharmingen (San Diego, CA): Cy-Q-labelled mouse anti-rat CD4 (clone OX-35), fluorescein isothiocyanate (FITC)-labelled mouse anti-rat CD3 (clone G4.18), phycoerythrin (PE)-labelled hamster anti-rat TNF $\alpha$  (clone TN-19.12) and PE-labelled hamster IgG isotype control (clone G235-2356).

**2.2.2.3. Incubation.** Immediately after sampling, 200 µl of heparinised whole blood was mixed with 200 µl of RPMI and stimulated with LPS (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 µM) was added to enable accumulation of TNF $\alpha$  in the Golgi complex by interrupting intracellular transport processes. Samples were incubated for 4 h at 37 °C and 5% CO<sub>2</sub>.

**2.2.2.4. Sample labelling.** After incubation, both the stimulated and the unstimulated sample were aliquoted into two tubes (200 µl per tube) and incubated with 4 µl  $\alpha$ -CD4 and 4 µl  $\alpha$ -CD3 at saturating dilutions for 30 min. Thereafter, red blood cells were lysed by adding 1 ml of lysing buffer to the tubes. After 5-min incubation at room temperature in the dark, all tubes were centrifuged and aspirated. The pellets were washed once with 2 ml ice-cold washing buffer. The remaining pellet was resuspended in 200 µl freezing buffer and stored at –80 °C until all samples of one rat were collected.

When all samples of one rat were collected, the samples were thawed and washed with ice-cold washing buffer once. Before permeabilisation with saponin, cells were fixed in 1 ml fixation buffer for 10 min. After centrifugation and aspiration, the pellet was resuspended in saponin solution to permeabilise WBCs. After incubation for 30 min, the tubes were centrifuged and aspirated. Then, one unstimulated tube and one stimulated tube were incubated with 4 µl TNF $\alpha$  at a saturating dilution, while the other tubes were incubated with the isotype control at the same dilution. After incubation for 30 min, cells were washed with saponin buffer and then fixed with 100 µl fixation buffer. They were kept in dark at 4 °C until measurement by flow cytometry within 24 h.

**2.2.2.5. Flow cytometry.** Cells were analysed with the Coulter Epics Elite flow cytometer (argon ion 488-nm laser; Beckman-Coulter, UK). Two thousand monocytes were acquired whilst gating on CD3 $^-$ /CD4 $^+$  cells (i.e. monocytes) and data were saved for later analysis. Analysis was performed using Winlist 32 (Verity Software House, Inc., Topsham, ME).

**2.2.2.6. Data analysis.** During analysis, in a forward scatter-side scatter plot, a gate was set around the leukocytes (see Fig. 1; left graph, G1). This gate was then copied to a CD3/CD4 scatter plot and a second gate was set on CD3 $^-$ /CD4 $^+$  monocytes (Fig. 1; middle graph, G2). For these monocytes, a single parameter histogram was defined to evaluate the percentage of monocytes producing intracellular TNF $\alpha$  (Fig. 1; right graph); using the unstimulated control sample, a linear gate was set so that 99% of the

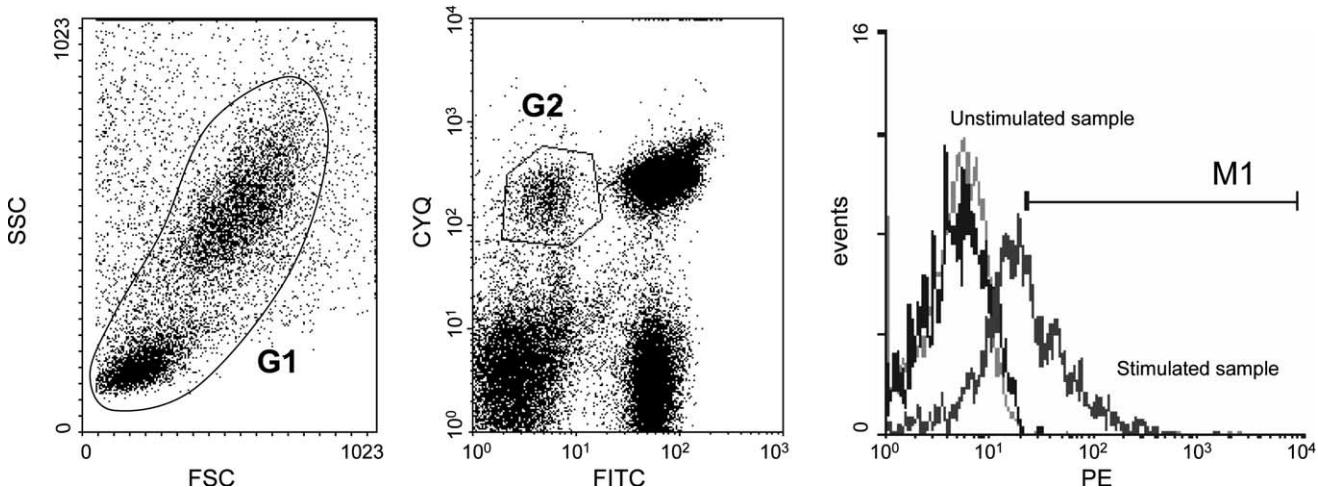


Fig. 1.  $\text{TNF}\alpha$  production of monocytes. After whole blood stimulation with LPS, granulocytes and lymphocytes can be demonstrated separately in a forward-scatter (FSC) and side-scatter (SSC) dot plot (left plot; gate G1); monocytes appear also in gate G1, they cannot be seen as a separate population. To analyse cytokine production by monocytes ( $\text{CD}3^-/\text{CD}4^+$  cells), the leukocytes in gate G1 were copied to a CD4-Cy-Q CD3-FITC dot plot (middle plot). Monocytes appear in gate G2. A single parameter fluorescence histogram was then used to evaluate the percentage of  $\text{TNF}\alpha$ -PE-labelled cells in this gate G2 (right plot). Using the unstimulated control sample, a linear gate (M1) was set so that 99% of the cells in the unstimulated sample were negative for cytokine production; this gate was then copied to the histogram of the stimulated cells. Results are expressed as percentage of positive cells in the stimulated sample (gray line represents the isotype control).

unstimulated cells were negative for TNF $\alpha$  (gate M1). This gate was copied to the histogram of the stimulated cells to evaluate the percentage of TNF $\alpha$ -producing monocytes after LPS stimulation as well as mean fluorescence intensity of the positive cells. The gray line represents the isotype control. The present method of selecting monocytes using CD3 and CD4 provided us with an extra internal negative control: CD3 $^{+}$ /CD4 $^{+}$  helper lymphocytes and CD3 $^{+}$ /CD4 $^{-}$  cytotoxic lymphocytes, which are not supposed to respond to LPS, were consistently negative for TNF $\alpha$  production in the stimulated samples.

### 2.2.3. Effect of freezing upon monocyte TNF $\alpha$ production

We have performed three separate experiments to evaluate the effect of freezing upon monocyte TNF $\alpha$  production. Therefore, blood was taken from a rat in the follicular phase and stimulated with endotoxin as described above. In each experiment, two stimulated and two unstimulated samples were frozen and treated as described above, and two stimulated and two unstimulated samples treated as above; however, after incubation with FLS, samples were centrifuged and aspirated, and immediately incubated with saponin. Samples were further treated as described above. The results showed no effect of freezing (percentage of TNF $\alpha$ -producing monocytes with freezing:  $30 \pm 3$ ; without freezing:  $31.5 \pm 3.5$ ). This is in line with our experiments in human (Bouman et al., 2001).

### 2.2.4. Statistics

Results are expressed as mean  $\pm$  standard error of mean (S.E.M.). Since the present study was a longitudinal study, in which rats were first tested during the 4 days of the oestrus cycle, after which the rats were rendered pregnant or pseudopregnant and testing was continued, paired testing was used. Therefore, for WBC counts, differential cell counts and percentage of TNF $\alpha$ -producing monocytes differences between the 4 days of the oestrus cycle, and differences between pregnancy/pseudopregnancy and the oestrus cycle, were first analysed with analysis of variance for paired samples, followed by a Wilcoxon's signed rank test to evaluate individual differences. Differences were considered significant if  $P < 0.05$ .

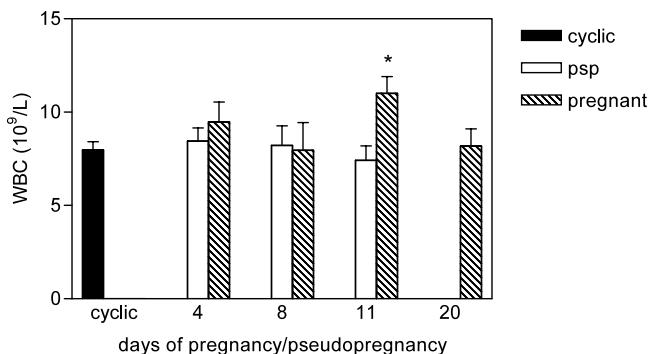


Fig. 2. Total number of WBCs in cyclic (black bar), pregnant (striped bars) and pseudopregnant (open bars) rats. \*, Significantly increased from cyclic rats (Wilcoxon's signed rank test,  $P < 0.05$ ).

### 3. Results

#### 3.1. Peripheral WBC counts

Fig. 2 shows the WBC counts of cyclic rats (black bar), pregnant rats (striped bar) and pseudopregnant rats (open bars). Since no differences were found in WBC counts between the 4 days of the oestrus cycle (analysis of variance for paired samples,  $P > 0.05$ ), the black bar represents the mean WBC counts of the 4 days of the oestrus cycle. It can be seen from this figure that pseudopregnancy did not affect peripheral WBC counts in the rat. During pregnancy, on the other hand, WBC counts were significantly increased, only on day 11, compared with cyclic rats (Mann–Whitney  $U$ -test,  $P < 0.05$ ).

The percentage of polymorphonuclear (PMN) (top panel), lymphocytes (middle panel) and monocytes (bottom panel), measured by flow cytometry, can be seen in Fig. 3. Percentages of PMN, lymphocytes and monocytes did not change during the 4-day follicular phase (analysis of variance for paired samples,  $P > 0.05$ ); therefore, the black bar in each panel represents the mean percentages of the 4-day oestrus cycle. Percentages of PMN, lymphocytes or monocytes did not change in pseudopregnant rats compared to cyclic rats. During pregnancy, the percentage of PMN was significantly increased on days 11 and 20, while the percentage of lymphocytes was significantly decreased on these days. No effect of pregnancy was observed upon the percentage of monocytes.

Results for percentage granulocytes, monocytes and lymphocytes as counted using the smears were similar to the results measured with flow cytometry, and therefore not shown.

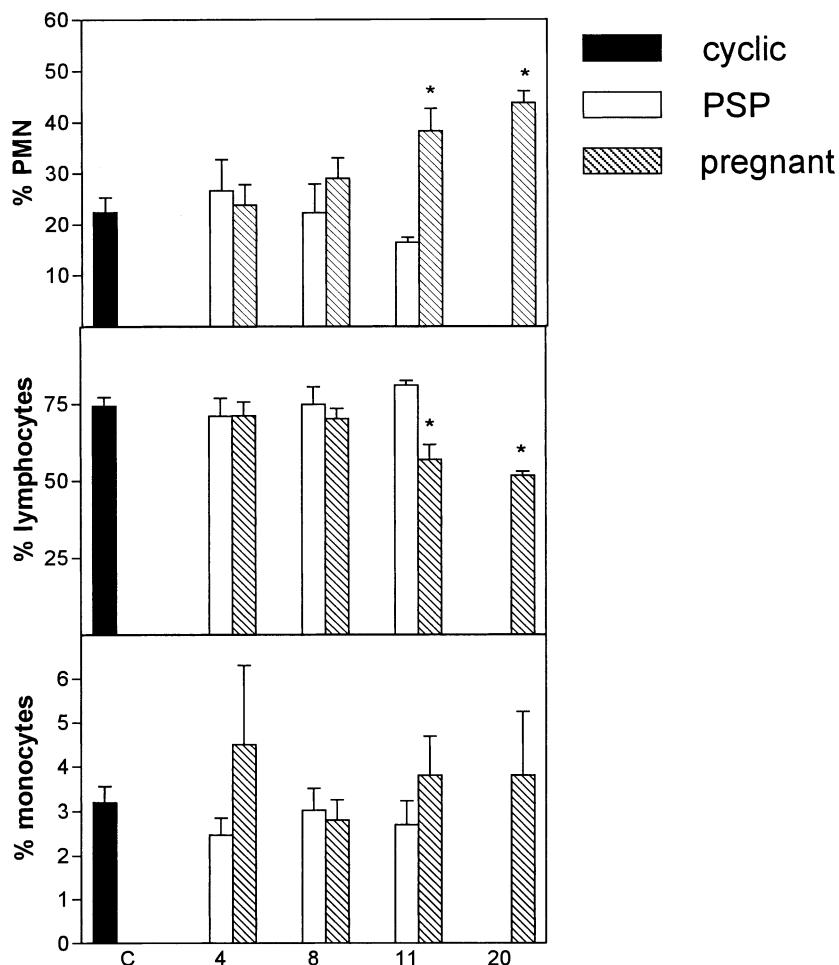


Fig. 3. Percentages of PMN (top panel), lymphocytes (middle panel) and monocytes (bottom panel) for cyclic rats (black bars), pregnant rats (striped bars) and pseudopregnant rats (open bars). \*, Significantly different from cyclic rats (Wilcoxon's signed rank test,  $P < 0.05$ ).

### 3.2. Monocyte TNF $\alpha$ production

Fig. 4 shows the percentage of monocytes producing TNF $\alpha$  after an in vitro LPS stimulus. The percentage of monocytes producing TNF $\alpha$  after in vitro LPS did not vary during the oestrus cycle (analysis of variance for paired samples,  $P > 0.05$ ); the black bar thus represents mean percentage of positive cells for the 4 days of the cycle. It can be seen that the percentage of monocytes producing TNF $\alpha$  after LPS stimulation was significantly increased in pseudopregnant rats compared with cyclic rats on all days

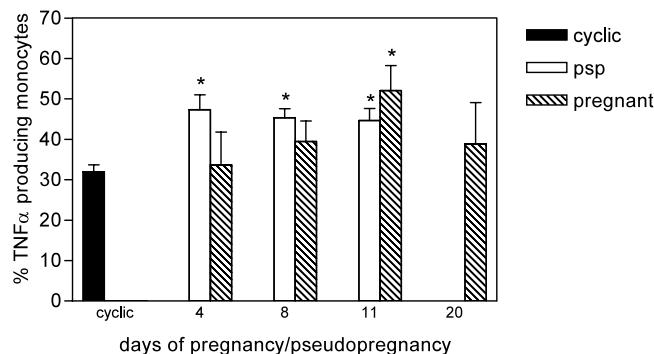


Fig. 4. Percentage of TNF $\alpha$ -producing monocytes after in vitro LPS stimulation of whole blood of cyclic rats (black bar), pregnant rats (striped bars) and pseudopregnant rats (open bars). \*, Significantly increased from cyclic rats (Wilcoxon's signed rank test,  $P < 0.05$ ).

measured. During pregnancy, the percentage of TNF $\alpha$ -producing monocytes was significantly increased only at day 11 of pregnancy compared with the oestrus cycle.

No effect upon mean fluorescence intensity of the positive cells, a measure of the amount of TNF $\alpha$  per cells, of either pregnancy or pseudopregnancy was observed (results not shown).

#### 4. Discussion

The present study was set up to evaluate monocyte function of rats in various reproductive conditions. Since one of the main functions of monocytes is to produce cytokines, so that they can communicate with other immune cells, we used LPS-induced (intracellular) TNF $\alpha$  production as a parameter of monocyte function. We showed that on days 4, 7 and 11 of pseudopregnancy, the percentage of monocytes producing TNF $\alpha$  after an LPS stimulus is increased compared to the follicular phase of the ovarian cycle in rats (i.e. cyclic rats), while during pregnancy the percentage of monocytes producing TNF $\alpha$  after an LPS stimulus is increased on day 11 of pregnancy, not on days 4, 7 and 20. Monocytes thus appear to be more sensitive to LPS at the post-implantation phase of pregnancy and during pseudopregnancy. Since TNF $\alpha$  is the main mediator of the LPS effect in vivo (Cybulsky et al., 1988), the increased TNF $\alpha$  production during the post-implantation phase of pregnancy and pseudopregnancy, as shown in the present study, may explain the increased inflammatory response to LPS infusion during this phase of pregnancy and pseudopregnancy (Faas et al., 1995, 1997).

For the evaluation of TNF $\alpha$  production by monocytes, we measured intracellular monocyte TNF $\alpha$  production. To the best of our knowledge, this is the first paper to describe intracellular cytokine production by monocytes in the rat. The measurement of intracellular cytokine production of monocytes allowed us to measure cytokine production at the single-cell level. Whole blood stimulation was used, since the isolation of monocytes from whole blood may cause activation and artificial differences in monocyte responses, which are not present *in vivo* (Macey et al., 1995; Sacks et al., 1997). The method we used for measuring intracellular cytokine production was adapted from the method we used recently for measuring intracellular cytokine production in human monocytes (Bouman et al., 2001). This adapted method appeared to be extremely useful for measuring monocyte function in the rat.

Although the percentage of monocytes producing TNF $\alpha$  is increased during pseudopregnancy, the production of TNF $\alpha$  per cell is not affected during pseudopregnancy. This may be due to the fact that the dose of endotoxin used in the present study was chosen for its capacity to stimulate follicular phase monocytes maximally. Apparently, the TNF $\alpha$  production in the follicular phase is the maximal capacity of the monocytes. The increased percentage of TNF $\alpha$ -producing monocytes during pseudopregnancy in the rat is in line with similar experiments in human: an increased percentage of monocytes from the human luteal phase produce TNF $\alpha$  upon *ex vivo* LPS stimulation compared with monocytes from the follicular phase (Bouman et al., 2001).

The question about the mechanism of the increased percentage of TNF $\alpha$ -producing monocytes during the post-implantation phase of pregnancy and pseudopregnancy remains. Differences between the follicular phase and pseudopregnancy in monocyte cytokine production upon LPS stimulation may be induced by increased progesterone and oestradiol concentrations during pseudopregnancy (Bouman et al., 2001). On the other hand, however, we have previously shown that developing ovarian follicles, only present during the follicular phase of the ovarian cycle, may produce anti-inflammatory factors (Schuiling et al., 2000). Therefore, it can also be suggested that differences between the follicular phase and pseudopregnancy in monocyte cytokine production upon LPS stimulation may be the result of anti-inflammatory factors produced by developing ovarian follicles in the follicular phase. This is currently under investigation in our laboratory.

Although in both pregnancy and pseudopregnancy the percentage of TNF $\alpha$ -producing monocytes was increased compared with the follicular phase, the timing was different between the two reproductive conditions: during pseudopregnancy, the percentage of TNF $\alpha$ -producing monocytes was

increased from day 4 until the end of pseudopregnancy (day 11), while during pregnancy this increase was only apparent on day 11. Assuming that monocyte cytokine production is under control of ovarian factors (either progesterone or oestrogen or inhibitory follicular factors), and since these factors are similar in pregnant and pseudopregnant rats, monocyte TNF $\alpha$  production seems to be inhibited during the implantation phase of pregnancy. It has indeed been suggested that placental cells, i.e. trophoblast cells and maternal cells (for instance, decidual cells; Bobe et al., 1986; Clark et al., 1984), as well as factors produced by these cells in culture (Raghupathy, 1997), have immunomodulatory roles. The exact mechanism as well as the reason why this inhibition is released by day 11 cannot be deduced from the present experiments. It may, however, be related to the fact that on day 10 of pregnancy contact between fetal tissue and maternal blood is established (Welsh and Enders, 1991).

Next to differences in monocyte function between the three reproductive conditions, we also found differences in total number of WBCs as well as in percentages of PMN and lymphocytes. Total WBC count was significantly increased on day 11 of pregnancy compared with rats in the follicular phase of the ovarian cycle. This seemed to be due to increased numbers of PMN. The present data are not in line with the scarce data found in the literature (LaBorde et al., 1999; Papworth and Clubb, 1995), since in the two previous studies no effect of pregnancy upon WBC count was found. The difference between the two previous studies and present study is the fact that, in previous studies, blood was obtained under anaesthesia, while in the present study blood was obtained from the jugular vein cannula while the rats were awake. Our study, therefore, represents better the natural *in vivo* situation.

The increase in WBC counts in rat pregnancy is in line with what is known about human pregnancy since, also in human pregnancy, WBC count is increased and, like in the present study, this is due to an increase in granulocyte number (Veenstra van Nieuwenhoven et al., 2002). The increase in granulocyte number may also suggest a proinflammatory state of the inflammatory system during the post-implantation phase of rat pregnancy. In human, the increase in granulocytes during pregnancy may be due to increased oestrogen levels (Bain and England, 1975); indeed, increased granulocyte numbers have also been found during the luteal phase of the ovarian cycle in women (Bain and England, 1975; Mathur et al., 1979). In rats, we did not find an increase in WBC counts during pseudopregnancy; therefore, in the rat, the increased granulocyte and monocyte numbers appear to be specific for pregnancy and may result from the presence of the fetomaternal unit.

In summary, in the present study we have demonstrated an increase in TNF $\alpha$ -producing monocytes after ex vivo LPS stimulation of whole blood from pseudopregnant and day 11 pregnant rats compared to follicular phase rats. While the mechanism of increased sensitivity of monocytes to LPS remains to be investigated, the present results suggest that the innate immune response in the post-implantation phase of pregnancy and during pseudo-pregnancy is increased in sensitivity to proinflammatory stimuli. The fact, however, that the timing of increased monocyte TNF $\alpha$  production differs between pregnant and pseudopregnant rats, and the fact that WBC counts and numbers of granulocytes are only increased during pregnancy, suggests that the state of activity of the inflammatory system, although both inclined towards a proinflammatory state, differs between pregnancy and pseudo-pregnancy. This suggests thereby a role for the fetoplacental unit in regulating the state of activity of the inflammatory system during pregnancy.

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