Species differences in the effect of pregnancy on lymphocyte cytokine production between human and rat

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Abstract: In the present study, we evaluated whether lymphocyte cytokine production during human and rat pregnancy shifts toward T helper cell type 2 (Th2) cytokine production. Therefore, blood samples were taken during the follicular and luteal phase and during pregnancy in rats and humans. Whole blood was ex vivo-stimulated with phorbol 12-myristate 13-acetate and calcium ionophore and intracellular interferon-γ (IFN-γ) and interleukin (IL)-4 production, and the percentage of cells in the various lymphocyte populations was measured using flow cytometry. Rats and humans adapted their immune responses to pregnancy but have different strategies: During human pregnancy, the percentage of lymphocytes producing IFN-γ was decreased, and the percentage IL-4-producing lymphocytes was not affected. The rat adapts its immune response to pregnancy by decreasing the total number of the various lymphocyte populations, and the percentage of IFN-γ- or IL-4-producing lymphocytes was not affected or increased (% IFN-γ-producing cytotoxic lymphocytes). It is speculated that during rat pregnancy, there is no need to decrease the number of IFN-γ-producing lymphocytes, as in nonpregnant rats, the total number of IFN-γ-producing lymphocytes after stimulation is relatively low, and there is no necessity for a further decrease. In nonpregnant humans, the percentage IFN-γ-producing lymphocytes is much higher and probably dangerous for pregnancy, and therefore, this percentage needs to decrease during pregnancy. In conclusion, although the data from humans concur with the Th1/Th2 paradigm, the data from rats do not concur with this paradigm. The present studies therefore challenge the classical Th1/Th2 paradigm during pregnancy. J. Leukoc. Biol. 78: 946–953; 2005.

Key Words: *interferon-γ · interleukin-4 · NK cells · luteal phase · follicular phase*

INTRODUCTION

It is well-known that pregnancy is associated with changes in the peripheral immune response. It seems likely that these changes are necessary to accommodate the semiallogeneic blastocyst. The general accepted idea has always been that type 1 cytokines, which are involved in cellular immune responses, are harmful for pregnancy, whereas type 2 cytokines, which are involved in humoral immune responses, are protective for the fetus [1]. This may, however, be an oversimplification, as it has been shown that the type 1 cytokine interferon-γ (IFN-γ) has a dual effect: It is not only harmful for pregnancy but also mandatory for the implantation of the blastocyst [2].

Still, various in vivo studies have shown that peripheral injection of IFN-γ during pregnancy causes abortion, and the type 2 cytokine interleukin (IL)-4 does not affect pregnancy [3]. In line with this, it has been shown by various groups that during human pregnancy, the ratio of the production of type 1 cytokines (IFN-γ and IL-2) and type 2 cytokines of peripheral helper and cytotoxic lymphocytes and natural killer (NK) cells is decreased during pregnancy [1, 4–6]. Whether this is a result of a decrease in type 1 cytokines or an increase in type 2 cytokines or both remains controversial [1, 6].

Also, during rat pregnancy, immune responses change. We have previously shown that peripheral lymphocytes, monocytes, and granulocytes show an activated phenotype, especially in the last week of rat pregnancy [7]. Moreover, total white blood cells (WBC) counts as well as endotoxin-induced monocyte tumor necrosis factor α production are increased during pregnancy [8]. However, no data are available showing that in rat pregnancy, like in human pregnancy, the balance between T helper cell type 1 (Th1) and Th2 cytokines has shifted toward type 2 cytokine production.

We therefore conducted a study in which we evaluated the effect of pregnancy on lymphocyte and NK cell IFN-γ and IL-4 production in human and rat. Moreover, also, women in the luteal phase of the ovarian cycle and rats with an induced luteal phase (pseudopregnant rats) were included in our study to evaluate whether possible effects of pregnancy were a result of the increased progesterone and oestradiol concentrations; levels of these ovarian hormones are increased during pregnancy and the luteal phase.

MATERIALS AND METHODS

Experimental design

In humans, blood samples were taken in the spontaneous ovarian cycle as described previously [9]: the first sample, 6–9 days after the first day of menstruation and the second sample, 6–9 days after a positive urinary luteinizing hormone (LH) test. Moreover, blood samples were taken at Week 30 of pregnancy in a second group of women with normal pregnancies as described previously [1]. Cyclic rats, i.e., rats in the follicular phase, were equipped with a jugular vein cannula for stress-free blood sampling, and blood samples were taken on dioestrus of the spontaneous ovarian cycle of the rats, which is mid-follicular phase, after which the rats were rendered pregnant, or a luteal phase was induced, and blood samples were taken at Day 16 of pregnancy (pregnancy in the rat lasts 21 days; as a consequence, Day 16 is similar to the third trimester of human pregnancy) and Day 7 of this luteal phase (this induced luteal phase in the rat lasts for 12 days; therefore, Day 7 is similar to mid-luteal phase in humans). In humans and rats, immediately after sampling, blood samples were stimulated with phorbol 12-myristate 13-acetate (PMA) and calcium (Ca) ionophore, and intracellular production of IFN-γ and IL-4 was measured using flow cytometry.

List of reagents used for cell activation and cell staining

The following reagents were used: Brefeldin A (eBioscience, San Diego, CA), monensin (Sigma Chemical Co., St. Louis, MO), FACS™ lysing solution (Becton Dickinson Immunocytometry Systems, San Jose, CA), fluorescein-activated cell sorter (FACS) permeabilization solution (Becton Dickinson Immunocytometry Systems), PMA (Sigma Chemical Co.), Ca ionophore A-23187 (Sigma Chemical Co.), washing buffer [phosphate-buffered saline (PBS) with 0.5% bovine serum albumin and 0.1% NaN_3], saponin solution (0.5% in washing buffer), complete RPMI-1640 medium (Gibco-BRL, Breda, The Netherlands), supplemented with 60 μg/ml gentamycin, and fixation buffer (0.5% paraformaldehyde in PBS).

Human subjects

Institutional approval was obtained from the local ethics committee, and all subjects signed informed consent. For all subjects, exclusion criteria were evidence of treatment with antibiotics, flu-like symptoms within 14 days of blood sampling, as well as the presence of any known diseases.

We included 15 women with regular menstrual cycles (cycle length between 26 and 32 days and age 31±4). The first sample in these women was taken in the mid-follicular phase, 6–9 days after the first day of menstruation. The second sample was obtained in the mid-luteal phase, 6–9 days after a positive urinary LH test {Clindia Benelux, b.v., Leusden, The Netherlands; data of part of these women (n=7) were used in our previous study [9]}.

Pregnant women (n=23) were recruited from our out-patient clinic, and blood samples were taken at 30 weeks amenorrhea {mean age 30±6, and all women were nulliparous; data of part of these women (n=9) were used in our previous study [1]}. Blood samples (20 ml) were obtained in vacutaner tubes (Becton Dickinson Immunocytometry Systems); one containing sodium heparin was used to evaluate intracellular cytokine production, and the other, containing EDTA, was used for measuring WBC counts.

Rats

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 ~200 gr) 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 (i.e., rats in the follicular phase of the ovarian cycle) were selected for the experiments. Twelve regularly cycling rats were equipped with a permanent jugular vein cannula under NO_2/fluothane anesthesia, according to the method of Steffens [10].

Pregnancy was achieved by housing the female rats on the night of proestrus 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 (or pseudopregnancy) [11], it was induced by electrical stimulation of the cervix uteri on proestrus (at 17.00 h) and on estrus (at

15.00 h), according to standard methods [12]. Plasma progesterone concentrations were measured on dioestrus in rats in the follicular phase and on Day 10 of the luteal phase (radioimmunoassay, according to the method of de Jong et al. [13]). Mean plasma concentration of progesterone was 35.1 ± 2.73 nM during the follicular phase and significantly increased to 120.1 ± 8.5 nM on Day 10 of pseudopregnancy, indicating that a luteal phase was achieved.

Blood samples (500 μl) were withdrawn from the permanent jugular vein cannula of all rats at dioestrus-2 in the follicular phase (n=12). After blood had been taken during the follicular phase, six of the rats were rendered pregnant; in the other six, a luteal phase was induced. Blood samples (500 μl) were taken on Day 16 of pregnancy and on Day 7 of the luteal phase. For all blood samples, 480 μl of the sample was collected in a sterile heparin vacutainer tube for measuring intracellular cytokine production; 20 μl of this blood was collected in EDTA for measuring WBC count.

Sample handling

WBC counts

For human and rat samples, 20 μL EDTA blood was used to determine total WBC counts, which were measured with a micro-cell counter (Model Sysmex F800, Toa Medical Electronics Co., Kobe, Japan).

Human samples

Antibodies

The following antibodies were used and (unless stated otherwise) purchased from IQ-Products (Groningen, The Netherlands). Cy-Q-labeled mouse anti-human CD3 (clone B-B11), fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD8 (clone MCD8), phycoerythrin (PE)-labeled mouse anti-human IFN-γ (clone 45-15), PE-labeled mouse anti-human IL-4 (clone 8F-12), FITC-labeled mouse anti-human CD94 (clone HP-3B1, PharMingen, San Diego, CA), and PE-labeled mouse isotype control immunoglobulin G1 (IgG1; clone MCG1).

Incubation

Immediately after sampling, 1 ml heparinized whole blood was mixed with 1 ml RPMI 1640 and stimulated with PMA (40 nM) and Ca ionophore (2 nM) for 4 h at 37°C and 5% CO_2. Whole blood (1 ml) was used as the unstimulated control and only mixed with 1 ml RPMI 1640. In all samples, monensin (3 μM) was added to enable accumulation of the cytokines in the Golgicomplex by interrupting intracellular transport processes.

Sample labeling

Sample labeling was done as described previously [9]. In brief, after 4 h incubation, all samples were aliquoted (0.2 ml per tube), red blood cells (RBC) were lysed, and WBC were permeabilized. Then, stimulated and unstimulated aliquots were incubated for 30 min at room temperature (RT) in the dark with anti-CD3 (5 μl), anti-CD8 (5 μl), and anti-IFN-γ, anti-IL-4, or isotype control at saturating dilutions. After antibody incubation, the cells were fixed with fixation buffer and kept at 4°C until flow cytometric analysis (within 24 h).

Rat samples

Antibodies

Antibodies were purchased from PharMingen: allophycocyanin-labeled anti-rat CD3, FITC-labeled anti-rat CD8a, PE-labeled mouse anti-rat IL-4, PE-labeled mouse anti-rat IFN-γ, and PE-labeled mouse IgG$_1$ κ isotype control.

Incubation

The protocol used to measure intracellular IFN-γ and IL-4 production in lymphocyte populations in the rat was adapted from the protocol used for measuring intracellular cytokine production by human lymphocytes as described above with minor modifications. Immediately after sampling, 290 μL heparinized whole blood was mixed with 290 μL RPMI 1640 and stimulated with PMA (40 nM) and Ca ionophore (2 nM); 190 μl whole blood was used as the unstimulated control and only mixed with 190 μl RPMI 1640. In the stimulated and the unstimulated samples, Brefeldin A (1 μg/100 μl) was added to enable accumulation of cytokines in the Golgicomplex by interrupting intracellular transport processes [14]; pilot experiments have shown that for rat lymphocytes, brefeldin A gives better results then monensin. Samples were incubated for 4 h at 37°C and 5% CO_2.

Sample labeling

After incubation, the stimulated sample was aliquoted into three tubes, and the unstimulated sample was aliquoted into two tubes (190 μl per tube); all tubes (stimulated and unstimulated) were incubated with 5 μl α-CD8 and 5 μl α-CD3 at saturating dilutions for 30 min. Thereafter, RBC were lysed by adding 1 mL lysing buffer to the tubes. After 5 min incubation at RT in the dark, all tubes were centrifuged and aspirated. The pellets were washed once with 2 mL ice-cold washing buffer. Then, cells were fixed in 1 mL fixation buffer for 10 min. After centrifugation and aspiration, the pellet was resuspended in saponin solution to permeabilize the WBC. In pilot experiments, it was found that saponin permeabilization gave better results than the FACS permeabilization solution. After incubation for 30 min, the tubes were centrifuged and aspirated. Then, the stimulated samples were incubated with 5 μl α-IFN-γ, 5 μl α-IL-4, or one with 5 μl isotype control at saturating dilutions, and the unstimulated samples were incubated with 5 μl α-IFN-γ or 5 μl α-IL-4 at saturating dilutions. After incubation for 30 min, cells were washed with saponin solution and then fixed with 100 μL fixation buffer. They were kept in the dark at 4°C until measurement by flow cytometry within 24 h.

Flow cytometry for human and rat samples

Cells were analyzed with the Coulter Epics Elite flow cytometer (argon-ion 488 nm laser, Beckman-Coulter, UK). Five thousand lymphocytes were acquired while life-gating on the lymphocyte population using forward- and side-scatter (FSC and SSC, respectively) characteristics, and data were saved for later analysis. Analysis was performed using Winlist 32 (Verity Software House, Topsham, ME). Moreover, for each sample, 1000 NK cells were acquired while life-gating on the CD3$^-$/CD94$^+$ population (human samples) or on the CD3$^-$/CD8^{dim} population (rat samples).

Data analysis

For human samples, during analysis in a FSC/SSC plot, a gate was set around the lymphocytes. This gate was then copied to a CD3/CD8 scatter plot, and a second gate was set on CD3$^+$/CD8$^-$ lymphocytes (T helper lymphocytes), on CD3$^+$/CD8$^+$ lymphocytes (T cytotoxic lymphocytes), or on CD3$^-$/CD94$^+$ (NK cells). For rat samples, during analysis in a FSC/SSC plot, a gate was set around the lymphocytes. This gate was then copied to a CD3/CD8 scatter plot, and a second gate was set on CD3$^+$/CD8$^-$ lymphocytes (T helper lymphocytes), CD3$^+$/CD8$^+$ lymphocytes (T cytotoxic lymphocytes), or on CD3$^-$/CD8^{dim} lymphocytes (NK cells).

For human and rat samples, for all these different lymphocyte populations, single-parameter histograms were defined to evaluate the percentage of lymphocytes producing intracellular IFN-γ or IL-4. Using the unstimulated control sample, a linear gate was set so that 99% of the unstimulated cells were negative for the cytokines IFN-γ or IL-4. This gate was copied to the histogram of the stimulated cells to evaluate the percentage of cytokine-producing lymphocytes after stimulation. The isotype control always overlapped the unstimulated sample, indicating that the lymphocytes in the unstimulated sample were not producing cytokines.

Differential blood cell counts

Using FSC and SSC characteristics, a gate was set around the total leukocyte population as well as on the lymphocyte population of an unstimulated sample. Percentage of lymphocytes was evaluated. Using the specific antibodies CD3 and CD8 (and CD94 for human samples) within the lymphocyte population, we evaluated the percentage of CD3$^+$, CD3$^+$/CD8$^+$, CD3$^+$/CD8$^-$, and CD3$^-$/CD8^{dim} (for rat samples) or CD3$^-$/CD94$^+$ cells (for human samples).

Statistics

Results are expressed as mean ± SEM. For human samples, data from the follicular phase and luteal phase were compared using Wilcoxon's signed rank test, and data from pregnancy were compared with data from the follicular phase using the Mann-Whitney U-test. For rat samples, paired testing was used for all data. Thus, data from pregnancy and data from luteal phase were compared with data from the follicular phase using the Wilcoxon's signed rank test. For all data, differences were considered significant if $P < 0.05$.

RESULTS

WBC counts

Figure 1 shows the total WBC counts of humans (left panel) and rats (right panel). The figure shows that in humans, WBC counts are increased during the luteal phase and pregnancy as compared with the follicular phase. In contrast, in rats, total WBC counts did not differ significantly among the follicular phase, luteal phase, and pregnancy.

Differential cell counts

The percentage of total lymphocytes within the total leukocyte population and percentage of T lymphocytes, T helper lymphocytes, T cytotoxic lymphocytes, and NK cells within the total
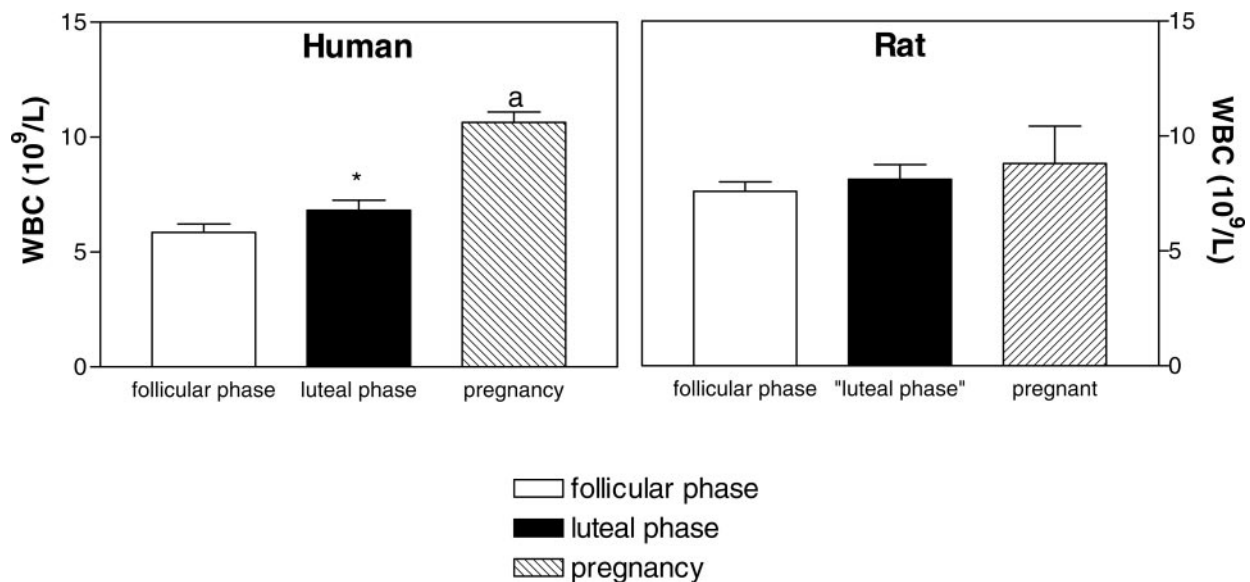

Fig. 1. Total WBC counts during the follicular phase (open bars), luteal phase (solid bars), and pregnancy (hatched bars) for humans (left panel) and for rats (right panel). *, Significantly different from the follicular phase (Wilcoxon's signed rank test, $P<0.05$); a, significantly different from the follicular phase (Mann-Whitney U-test, $P<0.05$).
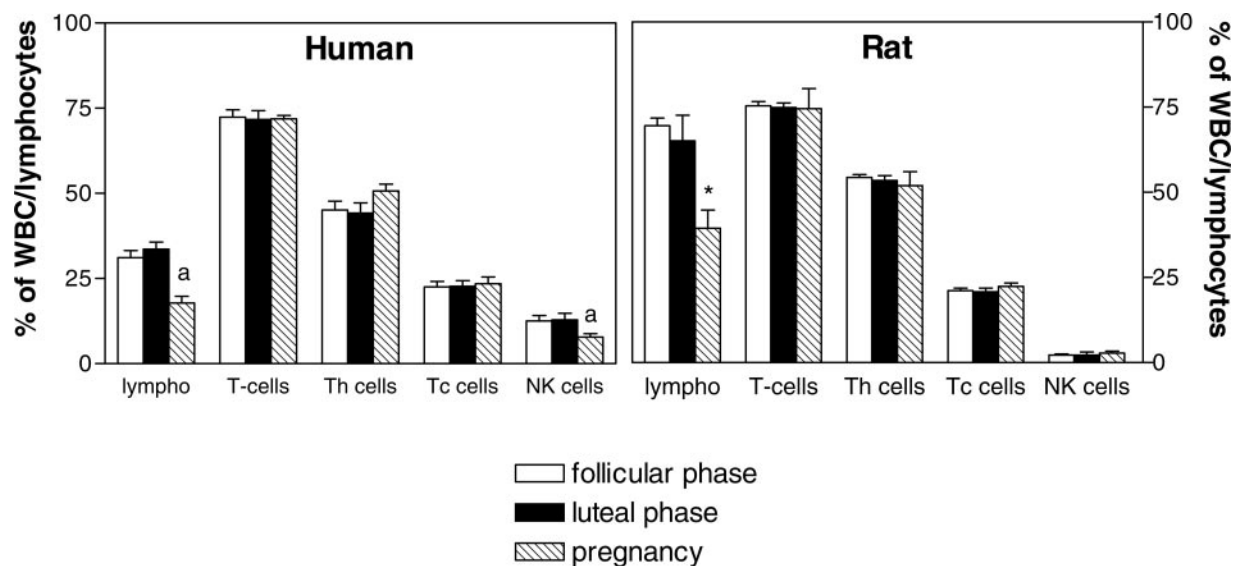
Fig. 2. Percentage of lymphocytes (lympho; left bars in both panels) from the total WBC population and percentage of T lymphocytes (T-cells), T helper lymphocytes (Th cells), T cytotoxic lymphocytes (Tc cells), and NK cells from the total lymphocyte population for humans (left panel) and rats (right panel). Open bars represent the follicular phase, solid bars represent the luteal phase, and hatched bars represent pregnancy. *, Signficantly different from the follicular phase (Wilcoxon's signed rank test, $P<0.05$); a, significantly different from the follicular phase (Mann-Whitney U-test, $P<0.05$).

lymphocyte population in humans and rats are shown in **Figure 2**. It is clear from this figure that the percentage of lymphocytes in the total leukocyte population is much higher in rats as compared with humans. However, the composition of the lymphocyte population is comparable between rats and humans. In humans and rats, the percentage of lymphocytes was decreased significantly during pregnancy. No effect of pregnancy or the luteal phase was seen on the percentage of T, T helper, or T cytotoxic lymphocytes in humans and in rats. The percentage of NK cells decreased significantly during pregnancy, only in humans and not in rats.

Percentage cytokine-producing cells

Figure 3 shows the percentage IFN-γ- and IL-4-producing lymphocytes for humans and rats. The difference in the percentage of IFN-γ- and IL-4-producing lymphocytes between humans and rats is clear: The percentage IFN-γ- and IL-4-producing lymphocytes is increased in all cell populations in humans as compared with rats. In humans, the percentage IFN-γ-producing lymphocytes is not affected by the luteal phase but decreased during pregnancy. The percentage of IL-4-producing T helper cells is increased during the luteal phase as compared with the follicular phase, and no effect was seen from pregnancy or the luteal phase on the percentage IL-4-producing cells in the other cell populations.

In contrast to the situation in humans, in the rat, the percentage of IFN-γ-producing T cytotoxic lymphocytes is increased rather then decreased in luteal phase and pregnancy, and the percentage of IFN-γ-producing NK cells is only increased during the luteal phase as compared with the follicular phase. No effect of pregnancy or luteal phase was seen on the percentage of IFN-γ-producing T helper cells, and also, no effect of pregnancy or luteal phase was seen on IL-4-producing cells in rats. Rat NK cells do not produce IL-4 in response to PMA and Ca ionophore.

Absolute numbers of circulating lymphocytes

It can be observed from **Figure 4**, which shows the absolute numbers of circulating peripheral lymphocytes per liter in humans and rats, that the absolute number of lymphocytes, T lymphocytes, T helper lymphocytes, and T cytotoxic lymphocytes is higher in rats in all reproductive phases as compared with humans. Also, the effect of the luteal phase and pregnancy upon lymphocyte numbers differed between rats and humans. Whereas in humans, lymphocyte, T, T helper, and T cytotoxic lymphocyte numbers and NK cell numbers are significantly increased during the luteal phase as compared with follicular phase with no difference between pregnancy and the follicular phase, in the rat, lymphocyte, T, T helper, and T cytotoxic lymphocyte numbers decreased significantly during pregnancy as compared with the follicular phase, with no effect of the luteal phase.

Absolute numbers of cytokine-producing cells

Absolute numbers of cytokine-producing cells per liter are shown in **Figure 5**. Numbers of IFN-γ-producing lymphocytes are decreased during human pregnancy as compared with the follicular phase. In contrast, in rats, no effect of pregnancy or luteal phase was observed on numbers of IFN-γ-producing lymphocytes.

Absolute numbers of IL-4-producing T helper and T cytotoxic lymphocytes are increased during pregnancy and luteal phase in humans. However, in rats, numbers of IL-4-producing T helper and T cytotoxic lymphocytes are decreased during the luteal phase and pregnancy.

DISCUSSION

Studies in humans have shown that during pregnancy, peripheral immune responses are changed as compared with peripheral immune responses in nonpregnant women. The present
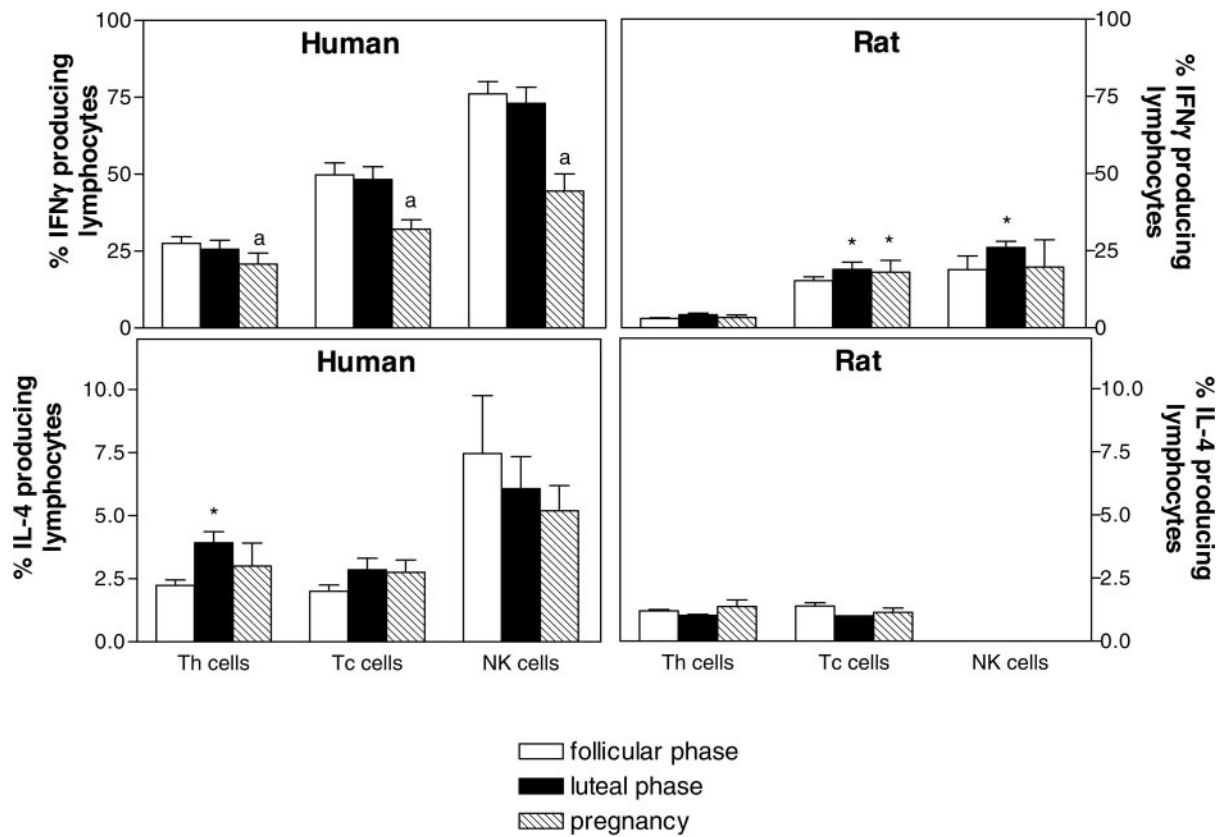
Fig. 3. Percentage of IFN-γ-producing lymphocytes (top panels) and percentage of IL-4-producing lymphocytes (bottom panels) for humans (left panels) and rats (right panels). In each panel, the first three bars represent T helper lymphocytes, the middle three bars represent T cytotoxic lymphocytes, and the last three bars represent NK cells. Open bars, Follicular phase; solid bars, luteal phase; and hatched bars, pregnancy. *, Signficantly different from the follicular phase (Wilcoxon's signed rank test, $P<0.05$); a, significantly different from the follicular phase (Mann-Whitney U-test, $P<0.05$).

study confirms and extends our previous study showing that the percentage of lymphocytes producing IFN-γ is decreased in pregnant women as compared with nonpregnant women, and the percentage of lymphocytes producing IL-4 did not differ between pregnant and nonpregnant women [1]. This results in a Th1/Th2 balance, which is shifted toward Th2 cytokine production [1]. We now show that the absolute numbers of IFN-γ-producing lymphocytes are decreased during human
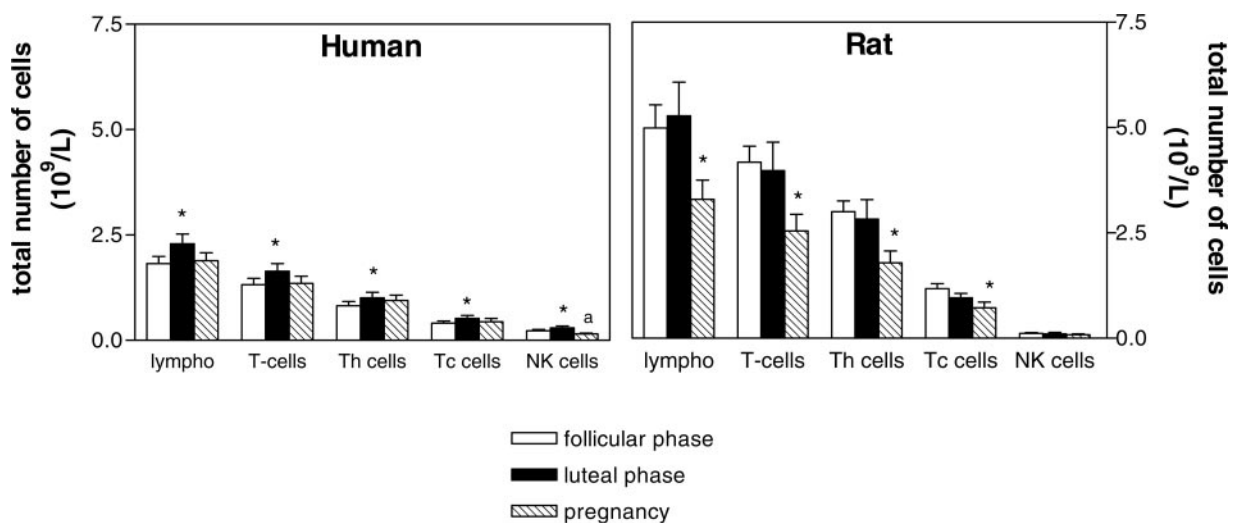

Fig. 4. Total number of the various lymphocyte populations per liter of blood for humans (left panel) and for rats (right panel). Presented lymphocyte populations: Lympho, Total lymphocyte population; T-cells, T lymphocytes; Th cells, T helper lymphocytes; Tc cells, T cytotoxic lymphocytes; and NK cells. Open bars represent follicular phase, solid bars represent the luteal phase, and hatched bars represent pregnancy. *, Signficantly different from the follicular phase (Wilcoxon's signed rank test, $P<0.05$); a, significantly different from the follicular phase (Mann-Whitney U-test, $P<0.05$).
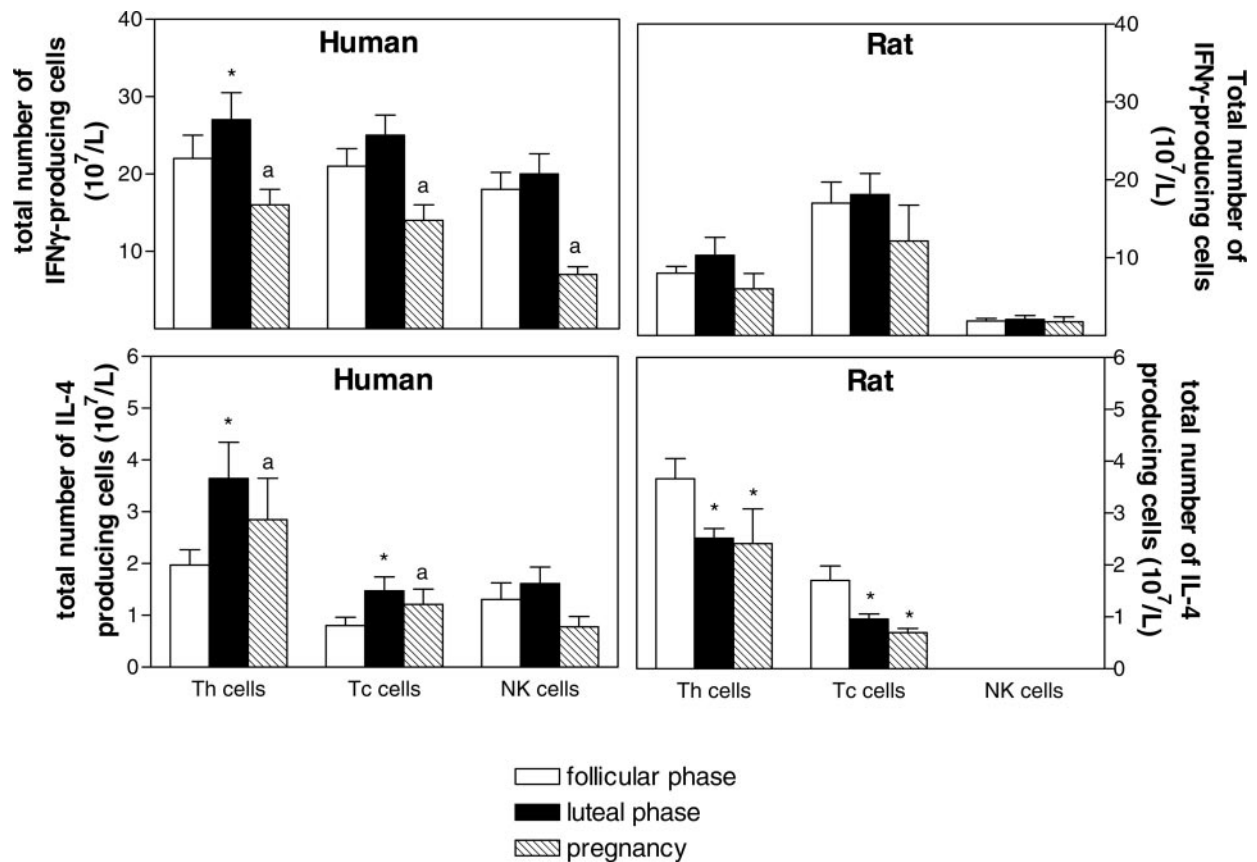
Fig. 5. Total number of IFN-γ-producing lymphocytes per liter (top panels) and total number of IL-4-producing lymphocytes per liter (bottom panels) for humans (left panels) and rats (right panels). In each panel, the first three bars represent T helper lymphocytes, the middle three bars represent T cytotoxic lymphocytes, and the last three bars represent NK cells. Open bars, Follicular phase; solid bars, luteal phase; hatched bars, pregnancy. *, Signficantly different from the follicular phase (Wilcoxon's signed rank test, $P<0.05$); a, significantly different from the follicular phase (Mann-Whitney U-test, $P<0.05$).

pregnancy as compared with the follicular phase but that absolute numbers of IL-4-producing cells are increased during pregnancy as compared with the follicular phase.

This is not only the first study to compare Th1/Th2 cytokine production in rat and human pregnancy; it is also the first study to measure Th1/Th2 cytokine production during rat pregnancy. Our data demonstrate that during human pregnancy, specific immune responses shift to a Th2 immune response, i.e., humoral immune response. Our data for human pregnancy are in line with data from other groups: There is a general consensus in the literature that the immune response during normal human pregnancy shifts toward a Th2 response [1, 6, 15–17]; there is, however, no consencus as to whether this is a result of a decreased Th1 response [1, 15, 16], such as shown by our own data, or an increased Th2 response [6] or both [17]. In contrast to human pregnancy, we found that during rat pregnancy, the immune response, as measured by the percentage of cytokine-producing lymphocytes, is shifted toward a Th1 immune response. This is a result of an increased percentage of T cytotoxic lymphocytes producing IFN-γ during pregnancy, and the percentage of IL-4-producing lymphocytes did not change. Therefore, the present study challenges the general concept of a shift from a Th1 immune response toward a Th2 immune response during pregnancy.

The reason for the differences in adaptations of immune responses to pregnancy between rat and human remains un-

known but may be related to the difference in total number of lymphocytes producing IFN-γ. We found a striking difference in total lymphocyte numbers between rat and human females in the follicular phase of the ovarian cycle: The percentage of lymphocytes of the WBC population is much higher in rats as compared with humans (~70% in rats and 30–35% in humans; Fig. 2), resulting in twice as much circulating lymphocytes in rats in the follicular phase as compared with humans in the follicular phase (Fig. 4). The percentages of lymphocytes producing IFN-γ after polyclonal stimulation, however, is much lower in rats as compared with humans, which results in a lower total number of IFN-γ-producing lymphocytes after polyclonal stimulation in nonpregnant rats as compared with nonpregnant human females (Fig. 5). During human pregnancy, the total number of IFN-γ-producing lymphocytes was decreased during pregnancy, and in the rat, total numbers of IFN-γ-producing lymphocytes did not change during pregnancy. One could speculate that to ensure a normal pregnancy, there is a maximum number of lymphocytes that may produce IFN-γ after stimulation. To achieve this maximum number in humans, the total number of lymphocytes producing IFN-γ needs to decrease during pregnancy, and in rats, there is no need to change the number of lymphocytes producing IFN-γ.

A similar line of reasoning can be set up for the percentage of NK cells producing IFN-γ after stimulation. The percentage of NK cells within the lymphocyte population is much higher in

nonpregnant humans than in nonpregnant rats (Fig. 2); the total number of circulating NK cells is also slightly higher in nonpregnant humans as compared with nonpregnant rats (Fig. 4). Although we found no effect of pregnancy on percentages of NK cells or percentage of NK cells producing IFN-γ in the rat, in humans, the percentage of NK cells and the percentage of NK cells producing IFN-γ were decreased significantly during pregnancy as compared with women in the follicular phase (Fig. 3). As far as data for human pregnancy are concerned, our data corroborate previous findings [1, 18, 19]. Evaluation of the total number of IFN-γ-producing NK cells (Fig. 5) showed that there is no effect of pregnancy on total number of IFN-γ-producing NK cells in rats, and in humans, the total number of IFN-γ-producing NK cells is decreased during pregnancy. The net result is that during pregnancy in rats and humans, the total numbers of IFN-γ-producing NK cells are much more similar, i.e., ~2 versus 7 10^7/L, respectively, than during the follicular phase (~2 vs. 18×10^7/L, respectively). This also suggests that during pregnancy, there is a maximum number of NK cells that should produce IFN-γ upon a stimulus.

The present data suggest different mechanisms for humans and rats to adapt immune responses to pregnancy. For humans, there may be an increased need to adapt immune responses, especially lymphocyte IFN-γ production, as the total number of IFN-γ-producing cells during the follicular phase cells is much higher in humans as compared with rats. We found that the human adaptations are to decrease the percentage of IFN-γ-producing cells, without affecting the number of lymphocytes and lymphocyte subpopulations. Our data, upon number of lymphoyctes and lymphocyte subpopulations, corroborate some previous data [1, 5, 16], and others, however, did find differences in subpopulations of lymphocytes in pregnant women as compared with nonpregnant women [20]. Rats, however, do adapt their immune responses, but they do so by decreasing the total number of lymphocytes and total numbers of the various lymphocyte subpopulations during pregnancy. This decrease of number of lymphocytes and subpopulations has been shown before [8, 21] Moreover, they even increase the percentage of lymphocytes producing IFN-γ, which results in no net effect of pregnancy on the absolute numbers of IFN-γ-producing cells. Still, total number of IFN-γ-producing cells is lower in rat pregnancy as compared with human pregnancy. It may be suggested that the increase in absolute numbers of IL-4-producing lymphocytes in humans is necessary to get an acceptable Th1/Th2 ratio for pregnancy in humans.

In the present study, we also included rats and humans in the luteal phase to evaluate the effect of sex hormones on changes seen during pregnancy. The most striking effect of rat pregnancy on immune responses is the decrease in numbers of lymphocyte subpopulations. This seems to be specific for rat pregnancy; it was not observed during the luteal phase, and therefore, this decrease is probably not a result of increased concentrations of progesterone and estrogen during rat pregnancy. The decreased numbers of IL-4-producing cells, however, may be a result of the increased concentrations of sex hormones, as the decreased numbers of IL-4-producing cells is seen during pregnancy and the luteal phase. Also, in human pregnancy, sex hormones seem to affect lymphocyte IL-4 production; however, Figure 5 shows that the total number of

IL-4-producing T helper and T cytotoxic cells is increased rather than decreased in pregnancy and in the luteal phase. This is in line with previous papers showing that progesterone increased stimulated IL-4 production after in vitro incubation of human cells [22].

Although the increased production of IL-4 during pregnancy and in the luteal phase may be explained by increased sex hormone concentrations, this is not the case for the decreased IFN-γ production during human pregnancy. In humans, the decreased percentage IFN-γ-producing cells is a result of a decreased number of IFN-γ-producing cells, which during pregnancy, is most likely not a result of estrogen or progesterone, as in the luteal phase, total numbers of IFN-γ-producing cells are increased (T helper cells) or decreased (T cytotoxic and NK cells). The lack of effect of sex steroids on IFN-γ production is in line with previous in vitro studies showing no effect of sex hormones on lymphocyte IFN-γ production [9, 22].

In summary, the present paper shows different adaptation strategies for immune responses during pregnancy in rats and humans. We show that although for human pregnancy, the classical Th1/Th2 paradigm is true, this is not the case for rat pregnancy. This study, therefore, challenges the classical Th1/Th2 paradigm during pregnancy. Also Zenclussen et al. [23], who showed decreased levels of IL-12, a strong Th1 cytokine inducer, in miscarriage patients compared with normal pregnant women, challenged this classical paradigm. Moreover, more data have become available to indicate that IFN-γ is not only detrimental for pregnancy but is also needed during pregnancy, for instance, during implantation of the blastocyst [2]. Based on our findings, we hypothesize that during pregnancy, there is a maximum percentage of cells producing IFN-γ to ensure a normal pregnancy. Depending on the species, the percentage of IFN-γ-producing cells slightly increases as compared with nonpregnant values (such as in the rat) or drammatically decreases from nonpregnant values (such as in humans).

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