

17 β -Estradiol and progesterone do not influence the production of cytokines from lipopolysaccharide-stimulated monocytes in humans

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Objective: To test whether 17 β -estradiol or progesterone influence the cytokine productive capacity of lipopolysaccharide (LPS)-stimulated monocytes in humans.

Design: Prospective study.

Setting: Academic research institution.

Patient(s): Seven women in the luteal phase of a normal ovarian cycle, 13 oral contraceptive users, 10 men, and 10 postmenopausal women.

Intervention(s): Blood samples collected from women in the luteal phase and from oral contraceptive users were used to study the *in vivo* effect of 17 β -estradiol and progesterone on monocyte cytokine production. Blood samples collected from men and postmenopausal women were used for *in vitro* incubation with 17 β -estradiol and progesterone.

Main Outcome Measure(s): The percentage of monocytes producing tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) after *in vitro* LPS-stimulation was determined.

Result(s): No *in vivo* relation in the productive capacities of LPS-stimulated monocytes in the luteal phase of the ovarian cycle was found between progesterone and TNF- α or IL-1 β ; or between 17 β -estradiol and TNF- α or IL-1 β . Moreover, the production of TNF- α and IL-1 β by LPS-stimulated monocytes did not vary between periods of oral contraceptive use and nonuse. The production of TNF- α and IL-1 β by LPS-stimulated monocytes in the blood of men and postmenopausal women *in vitro* was not influenced by incubation with different concentrations of 17 β -estradiol or progesterone.

Conclusion(s): We could not find evidence for a causal relationship between 17 β -estradiol or progesterone and TNF- α - or IL-1 β -production. We conclude that 17 β -estradiol and progesterone do not influence the cytokine-production capacity of LPS-stimulated monocytes in humans. (Fertil Steril® 2004;82(Suppl 3): 1212–1219. ©2004 by American Society for Reproductive Medicine.)

Key Words: Oral contraceptives, men, postmenopausal women, 17 β -estradiol, progesterone, immune response, TNF- α , IL-1 β , monocytes, cytokines

Although the interaction between the immune system and the reproductive system has long been a matter of interest, the precise correlation between sex hormone levels and immune function has yet to be identified. In previous research, we demonstrated that immune responses vary with the ovarian cycle in humans (1, 2). The specific immune response shifts toward a T_H2 response in the luteal phase as compared with the follicular phase (1). Moreover, we also found an increase in monocytes producing interleukin 1 β (IL-1 β) and tumor necrosis factor alpha (TNF- α) after *in vitro* activation by lipopolysaccharide (LPS) in the luteal phase as compared

with the follicular phase (2). This deviation of the immune response during the luteal phase may be necessary for the preparation of the maternal immune system for potential implantation of the semi-allogenic blastocyst (2). Therefore, it is necessary to study the regulation of this variation in immune response during the ovarian cycle.

The changes in immune responses during the luteal phase can result from increased concentrations of the sex hormones 17 β -estradiol and progesterone because the hormone concentration increases concomitantly with the devi-

Received December 30, 2003; revised and accepted May 7, 2004.

Supported by J.K. de Cock Foundation (Grant No. 00-12) and by an unrestricted educational grant from Organon, The Netherlands.

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0015-0282/04/\$30.00
doi:10.1016/j.fertnstert.2004.05.072

ation of the immune response in the luteal phase (2). On the other hand, differences in immune responses between the follicular and luteal phases may be a result of factors produced during the follicular phase—that is, one or more factors capable of suppressing the immune system during the follicular phase. Once the factor in question disappears (i.e., in the luteal phase), the nonspecific immune system is not suppressed anymore and thus appears to be “activated and more sensitive”; the specific immune system then shifts toward a T_H2 response (2).

In the present study, we focus on the nonspecific immune system; our experiments were designed to study the effect of 17β -estradiol and progesterone on the monocyte TNF- α and IL-1 β cytokine production in humans. In vivo, the effect of synthetic hormones on cytokine-production capacity of stimulated monocytes was measured in oral contraceptive (OC) users; also, the relation between 17β -estradiol and progesterone concentrations and the LPS-induced production of TNF- α and IL-1 β by monocytes was evaluated in women during the luteal phase. The in vitro effect of 17β -estradiol and progesterone on cytokine production was studied in the whole blood of two groups of healthy individuals: men and postmenopausal women.

Monocytes are the first cellular components of the nonspecific immune system to be activated by LPS; on activation, they produce various cytokines such as TNF- α and IL-1 β (3). Therefore, as in our previous study (2), cytokine production of monocytes after in vitro LPS stimulation was used as a parameter for the state of activity and sensitivity of the nonspecific immune response. The intracellular productive capacity of the peripheral blood monocytes of TNF- α and IL-1 β was measured by flow cytometry.

MATERIALS AND METHODS

Reagents for Cell Activation and Cell Staining

The reagents for cell activation and staining were monensin (Sigma Chemical, St. Louis, MO); FACS lysing solution (Becton Dickinson, Immunocytometry Systems, San Jose, CA); FACS permeabilizing solution (Becton Dickinson); complete RPMI 1640 (GIBCO BRL, Breda, The Netherlands) supplemented with 60 $\mu\text{g}/\text{mL}$ gentamicin; washing buffer (phosphate buffered saline with 0.5% bovine serum albumin and 0.1% NaN_3); and fixation buffer (0.5% paraformaldehyde in phosphate buffered saline).

Antibodies

The following monoclonal antibodies were used: fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD14 (clone UCHM1; IQ Products, Groningen, The Netherlands); phycoerythrin (PE)-labeled mouse anti-human TNF- α (clone Mab11; Pharmingen, San Diego, CA); PE-labeled mouse anti-human IL-1 β (clone AS10; Becton Dickinson); and PE-labeled mouse isotype control IgG1 (clone MCG1; IQ Products).

Participants

After we had obtained institutional review board approval and signed, written informed consent forms from participants, we took blood samples from seven healthy women who had regular menstrual cycles of lengths between 26 and 32 days (experiment 1), 13 healthy women who were using third-generation OCs (experiment 2), 10 healthy postmenopausal women, who were at least 1 year postmenopause (experiment 3), and 10 healthy men (experiment 4). The exclusion criteria for all experiments were evidence of treatment with antibiotics or flu-like symptoms within 14 days of the blood sample as well as the presence of any known diseases.

Sample Processing and Incubation

All blood samples (20 mL) were obtained in two Vacutainer tubes, one tube containing sodium heparin, the other ethylenediaminetetraacetate (EDTA). For all experiments the EDTA plasma was isolated after centrifugation of the sample at 3000 rpm. The plasma was frozen at -20°C until later hormone analysis (17β -estradiol, and progesterone) according to the method of Jurjens et al. (4) and de Jong et al. (5). Heparinized blood was used to evaluate intracellular cytokine productive capacity.

Experiment 1: Luteal Phase Blood Samples of Women with Normal Menstrual Cycles

In the women who had normal menstrual cycles, two blood samples were obtained during the luteal phase: the first sample was taken 3 days after a positive urinary leuteinizing hormone (LH) test (Clindia; Benelux b.v., Leusden, The Netherlands), and the second was taken 6 days after a positive urinary LH test. These days of the ovarian cycle were chosen because hormone concentrations increase from day 3 to day 6 of the luteal phase. Thus, we could study the effect of high and low hormone concentrations on LPS-stimulated monocyte cytokine production in the absence of a putative follicular factor.

Immediately after sampling, 0.5 mL of heparinized whole blood was mixed with 0.5 mL of RPMI and stimulated with 2 $\mu\text{g}/\text{mL}$ of LPS (*E. coli*, 0.55:B5, Whittaker) (stimulated sample). We used 0.5 mL of heparinized blood as an unstimulated control mixed only with 0.5 mL of RPMI. In both the stimulated and unstimulated samples, monensin (3 μM) was added to enable accumulation of the cytokines in the Golgi complex by interrupting intracellular transport processes. Stimulated and unstimulated samples were incubated for 4 hours at 37°C and 5% CO_2 . The 4-hour time period has been used in all our studies for a variety of reasons, including cell viability being 100% during this period, making it optimal for measurement of cells positive for IL-1 β and TNF- α .

Experiment 2: Blood Samples of Oral Contraceptive Users

In OC users, the first blood sample was obtained on the morning of the first day before starting OC (the pill-free period). The second and third samples were obtained on days 10 and 17, respectively, after starting a new OC cycle. Third-generation pills were used, composed of 30 or 35 μg of ethinylestradiol and a synthetic progestogen with low androgenicity (i.e., norgestimate, gestodene, or desogestrel). Immediately after sampling, the stimulation and the incubation processes took place, as already described for experiment 1.

Experiment 3 and 4: In Vitro Incubation of Whole Blood from Postmenopausal Women and Men for Progesterone and 17 β -Estradiol

To study the in vitro effects of 17 β -estradiol and progesterone on monocyte cytokine production, we used the whole blood of men and postmenopausal women. In vivo, these groups lack follicular growth and high concentrations of 17 β -estradiol and progesterone. One blood sample each was obtained from 10 postmenopausal women and 10 men. Of each group, five of the blood samples were used for the experiment with incubation of 17 β -estradiol, and the other five blood samples were used for the experiment with incubation of progesterone.

Stock solutions were made with a concentration of 10.61 mg of progesterone/mL ethanol or with a concentration of 0.392 mg of 17 β -estradiol/mL ethanol. Dilution series were made using saline and an albumin replacement (Cealb; Sanquin, CLB, Amsterdam, The Netherlands) in a 1:1 ratio.

Immediately after sampling, a series of tubes were filled with 0.3 mL of heparinized whole blood and mixed with 0.3 mL of RPMI and stimulated with 2 $\mu\text{g}/\text{mL}$ of LPS (stimulated samples). To one tube containing this mix no hormones were added (untreated sample); in the other tubes, 6 μL each of progesterone dilution or 17 β -estradiol dilution was added. The final concentrations in the tubes ranged from 1×10^{-5} to 1×10^{-9} mol/L (310 ng/mL to 31 pg/mL) progesterone, or 1×10^{-6} to 1×10^{-11} mol/L (270 ng/mL – 2.9 pg/mL) 17 β -estradiol.

To study the effect of ethanol per se, a dilution series was made for ethanol: 0.3 mL of heparinized blood was used as unstimulated control and only mixed with 0.3 mL of RPMI. In all samples, monensin (3 μM) was added to enable accumulation of the cytokines in the Golgi complex by interrupting intracellular transport processes. Stimulated and unstimulated samples were incubated for 4 hours at 37°C and 5% CO₂.

Sample Labeling for All Experiments

After incubation, both stimulated and unstimulated samples were aliquoted (0.2 mL per tube) and 5 μL anti-CD14 was added to each tube. Tubes were incubated at room

temperature in the dark for 30 minutes. Following incubation with 1 mL of lysing buffer for 5 minutes at room temperature in the dark, tubes were centrifuged and aspirated. The remaining pellets were resuspended in 0.5 mL of permeabilizing buffer and incubated in the dark for 10 minutes. The cells then were washed with 2 mL of ice-cold washing buffer. After centrifugation and aspiration, stimulated and unstimulated aliquots were incubated for 30 minutes in the dark at room temperature with either anti-TNF- α , anti-IL-1 β or isotype control (5 μL) at saturating dilutions. After washing with washing buffer, cells were fixed with fixation buffer and kept at 4°C in the dark until measured (within 24 hours).

Flow Cytometry

Cells were analyzed using the Coulter Epcis flow cytometer (argon ion 488-nm laser). A thousand monocytes were acquired while life-gating monocytes (CD14 positive cells) and were saved for later analysis. The analysis was performed using Winlist32 (Verity Software House, Inc., Topsham, ME).

Data Analysis of Intracellular Cytokines

During analysis, a gate was set on CD14⁺ monocytes (Fig. 1). A single parameter fluorescence histogram for the monocytes was defined for evaluation of intracellular cytokine production. For evaluation of cytokine production following in vitro LPS stimulation, unstimulated and stimulated aliquots were used. Using the unstimulated aliquot, we set a linear gate in the histogram so that at least 99% of the cells in this aliquot were negative for cytokine production (see Fig. 1). This gate was then copied to the histogram of the stimulated cells; results are expressed as percentage positive cells in the stimulated aliquots.

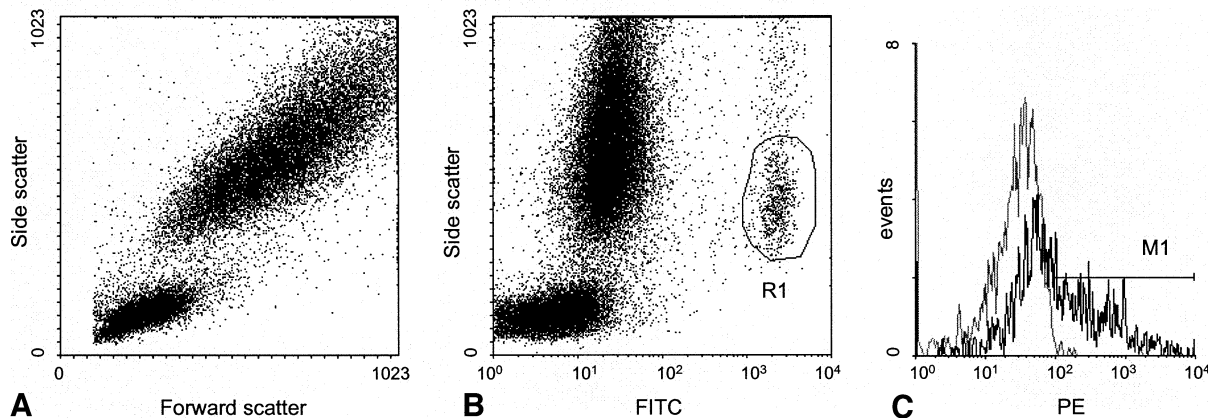
Statistics

Results in experiment 1 are expressed as the percentage positive monocytes per individual. To evaluate the relation between 17 β -estradiol- and progesterone-concentrations and the percentage of monocytes producing TNF- α - and IL-1 β in the luteal phase, we used a linear regression analysis (least sum of squares method). We calculated R^2 and the slope, and tested whether the slope was statistically significantly different from zero.

In experiment 2, the results are expressed as the mean percentage of positive monocytes \pm standard error of the mean (SEM). To evaluate differences between OC-free period and OC-use period, Wilcoxon's signed rank test was used. In experiment 3 and 4, to evaluate the effect of hormone incubations, the stimulated sample without hormone incubation was set at 100%. Samples with hormone incubations were related to this sample. Wilcoxon's signed rank test was used to evaluate differences in cytokine productive capacity of monocytes after incubation with different concentrations of 17 β -estradiol or progesterone. $P < .05$. was considered statistically significant.

FIGURE 1

CD14⁺ monocyte production of TNF- α . (A) After whole-blood stimulation with endotoxin (lipopolysaccharide), granulocytes and lymphocytes are demonstrated separately in a forward scattergram and sideward scattergram. (B) To analyze cytokine production by monocytes (CD14⁺ cells), monocytes were selected in a region R1 on a sideward scattergram and CD14-FITC dotplot. A single parameter fluorescence histogram was used to evaluate the number of phycoerythrin-positive cells in the monocyte R1 region. (C) Using the unstimulated control sample, linear gates were set in the histogram (M1) so that at least 99% of the unstimulated cells were negative for cytokine production. This gate was then copied to the histogram of the stimulated cells. Results are expressed as the percentage of positive cells in the stimulated blood sample (*gray line*, unstimulated; *black line*, stimulated sample).



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RESULTS

Participant Characteristics

The mean age of the women with a normal ovarian cycle was 31 years (range: 29 to 34 years). Their mean cycle length was 28 days (range: 26 to 32 days). Blood samples were taken at day 18 (range: 15 to 24 days) and day 21 (range: 18 to 27 days) of luteal phase. The mean age of the OC users was 28 years (range: 22 to 35 years), and all blood samples were taken at day 0, day 10, and day 17 of OC use. The mean age of the men was 34 years (range: 24 to 45). The mean age of the postmenopausal women was 56 years (range: 51 to 62).

Experiment 1: Women with Normal Menstrual Cycles

This study was set up to evaluate a possible relationship between plasma 17 β -estradiol and progesterone concentrations and LPS-induced monocyte cytokine production. To exclude a possible influence of a follicular factor, blood samples were taken only during the luteal phase. Figure 2 shows the individual percentages of monocytes producing IL-1 β (panels A and B) or TNF- α (panels C and D) in relation to the individual 17 β -estradiol and progesterone concentration of that same sample in the luteal phase of the normal ovarian cycle. The line represents the linear regression curve. As can be seen from the figures, progesterone or 17 β -estradiol concentrations had no effect on endotoxin-induced monocyte TNF- α or IL-1 β production.

Experiment 2: Oral Contraceptive Users

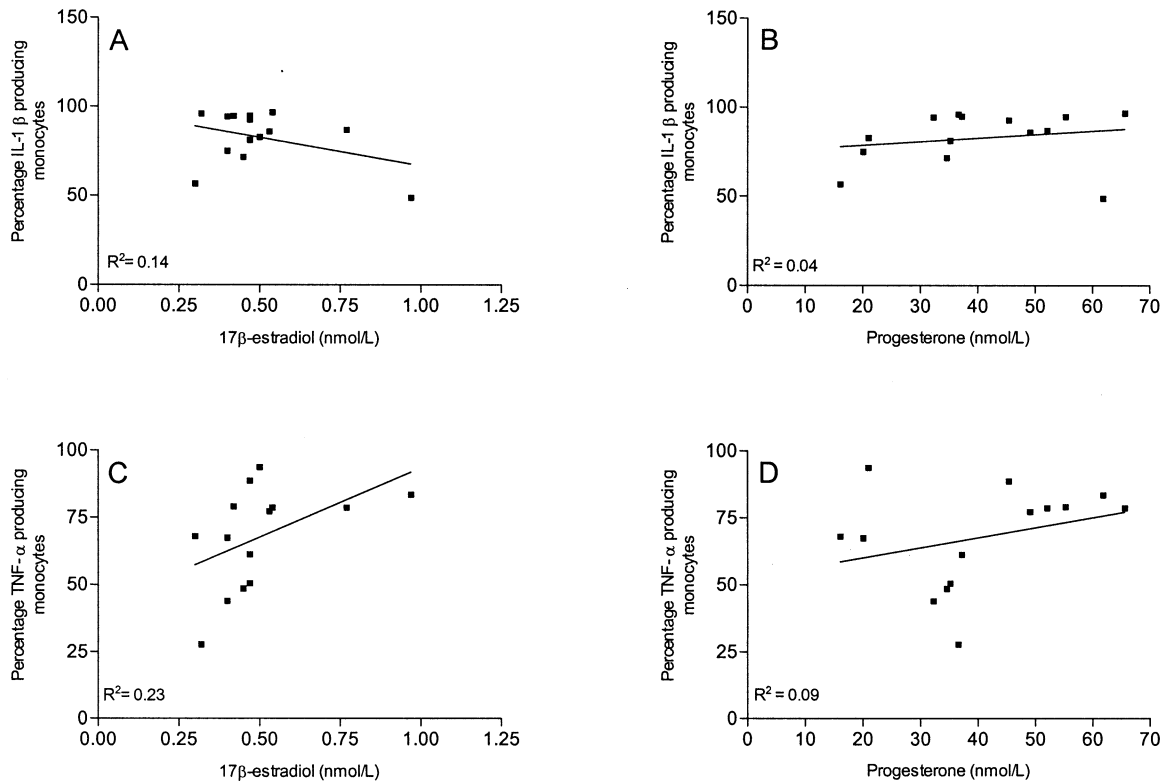
The 17 β -estradiol concentration in the OC-free period was statistically significantly higher compared with day 17 of OC use (0.49 nmol/L vs. 0.24 nmol/L, $P < .05$), but not compared with day 10 of OC use. No difference could be demonstrated in progesterone concentrations. Figure 3 shows the percentages of monocytes producing IL-1 β and TNF- α in the OC-free period and on days 10 and 17 of OC use. As can be seen from this figure, there is no statistically significant difference between the monocytes' capacity to produce IL-1 β and TNF- α in the OC free period when compared with days 10 or 17 of OC use.

Experiment 3: Postmenopausal Women

Figure 4 shows the percentage of monocytes producing IL-1 β - and TNF- α after incubation of whole blood of postmenopausal women with different concentrations of 17 β -estradiol and progesterone. Only at the concentration of 10⁻⁵ mol/L (310 ng/mL) of progesterone was any tendency toward decrease seen in the percentage of monocytes producing IL-1 β and TNF- α . This difference, however, was not statistically significant. As also can be seen, no effect of 17 β -estradiol concentrations could be found on the percentage of monocytes producing IL-1 β and TNF- α after LPS stimulation. Because no effect was seen from the ethanol dilution series on stimulated samples, we did not show these results in the figure.

FIGURE 2

(A, B) Individual percentages of monocytes producing IL-1 β in relation to (A) individual 17 β -estradiol concentrations and (B) progesterone concentrations in the same blood sample of women in the luteal phase of a normal ovarian cycle. (C, D) Individual percentages monocytes producing TNF- α in relation to (C) individual 17 β -estradiol concentrations and (D) progesterone concentrations in the same blood sample of women in the luteal phase of a normal ovarian cycle. (Lines = regression. Squares = individual sample. Slopes of regression lines do not differ from 0.)



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Experiment 4: Men

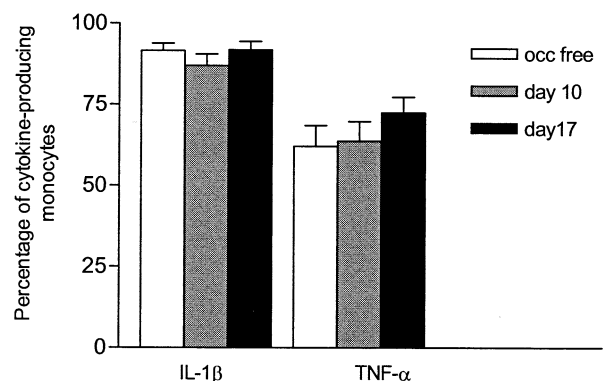
Figure 5 shows the percentage of monocytes producing IL-1 β and TNF- α after incubating the whole blood of men with different concentrations of 17 β -estradiol and progesterone. As can be seen, no effect of progesterone or 17 β -estradiol was found on the cytokine-production capacity of activated monocytes in men for either IL-1 β or TNF- α . Because no effect was seen from the ethanol dilution series on stimulated samples, we did not show these results in the figure.

DISCUSSION

In a previous study, we demonstrated an increased percentage of monocytes producing TNF- α and IL-1 β in the luteal phase as compared with the follicular phase (2). We suggested that this increase might be induced by the increase of 17 β -estradiol and progesterone in the luteal phase or by the absence of an inhibiting factor in the follicular phase. The present study was designed to study the effect of 17 β -estradiol and proges-

FIGURE 3

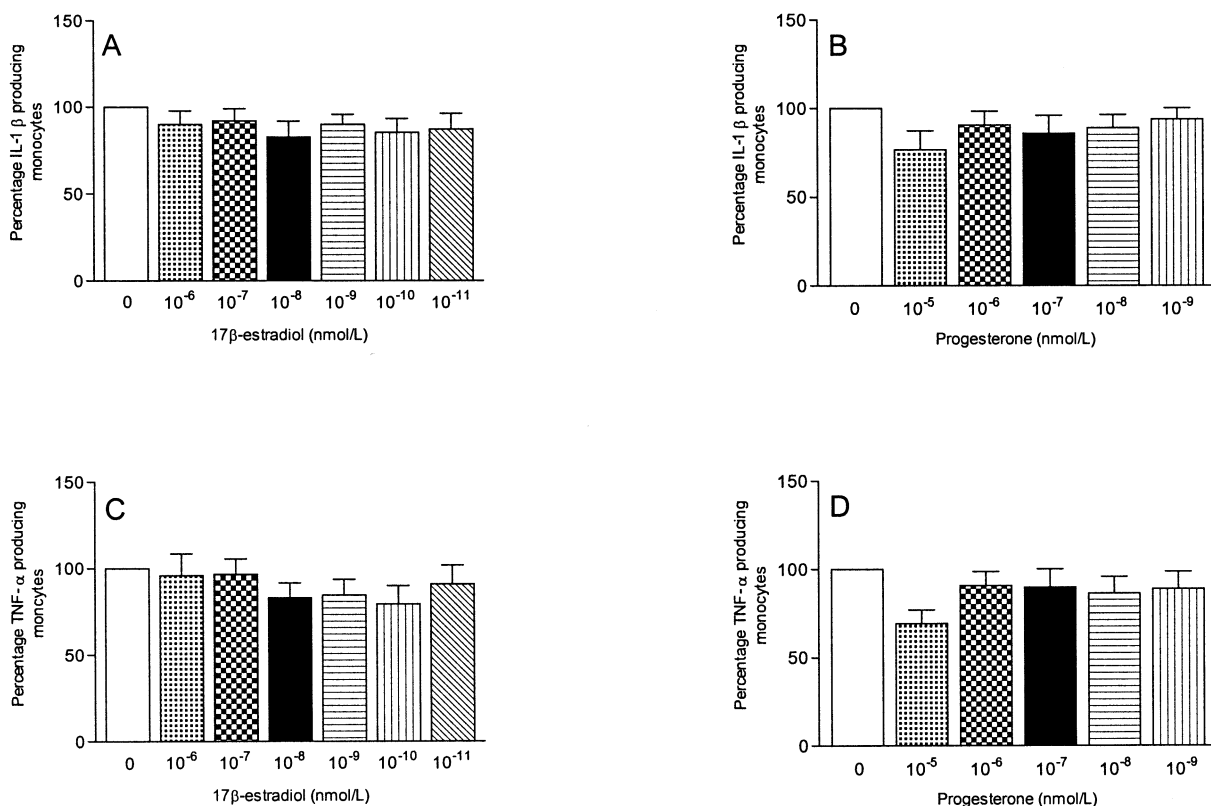
Mean (\pm SEM) percentage of monocytes producing IL-1 β and TNF- α during the period free of oral contraceptives and on days 10 and 17 of OC use.



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FIGURE 4

(A, B) Percentage of monocytes producing IL-1 β after in vitro incubation of whole blood of postmenopausal women, with different concentrations of (A) 17 β -estradiol and (B) progesterone. The stimulated sample not incubated with hormones was set to 100%. Hormone-incubated samples are related to this sample. (C, D) Percentage of monocytes producing TNF- α after in vitro incubation of whole blood of postmenopausal women, with different concentrations of (C) 17 β -estradiol and (D) progesterone. The stimulated sample not incubated with hormones was set to 100%. Hormone-incubated samples are related to this sample.



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terone in vitro and in vivo on the ability of monocytes to produce the cytokines TNF- α and IL-1 β in humans.

To evaluate the effect of 17 β -estradiol and progesterone on monocyte cytokine production in vitro, we used whole blood of men and postmenopausal women because, in both of these groups, there is no interaction with endogenous progesterone or 17 β -estradiol or a follicular factor. We found no effect of 17 β -estradiol or progesterone on the LPS-stimulated percentage of monocytes producing TNF- α . In the literature, conflicting data have been described about the influence of sex hormones in vitro on TNF- α production by stimulated monocytes.

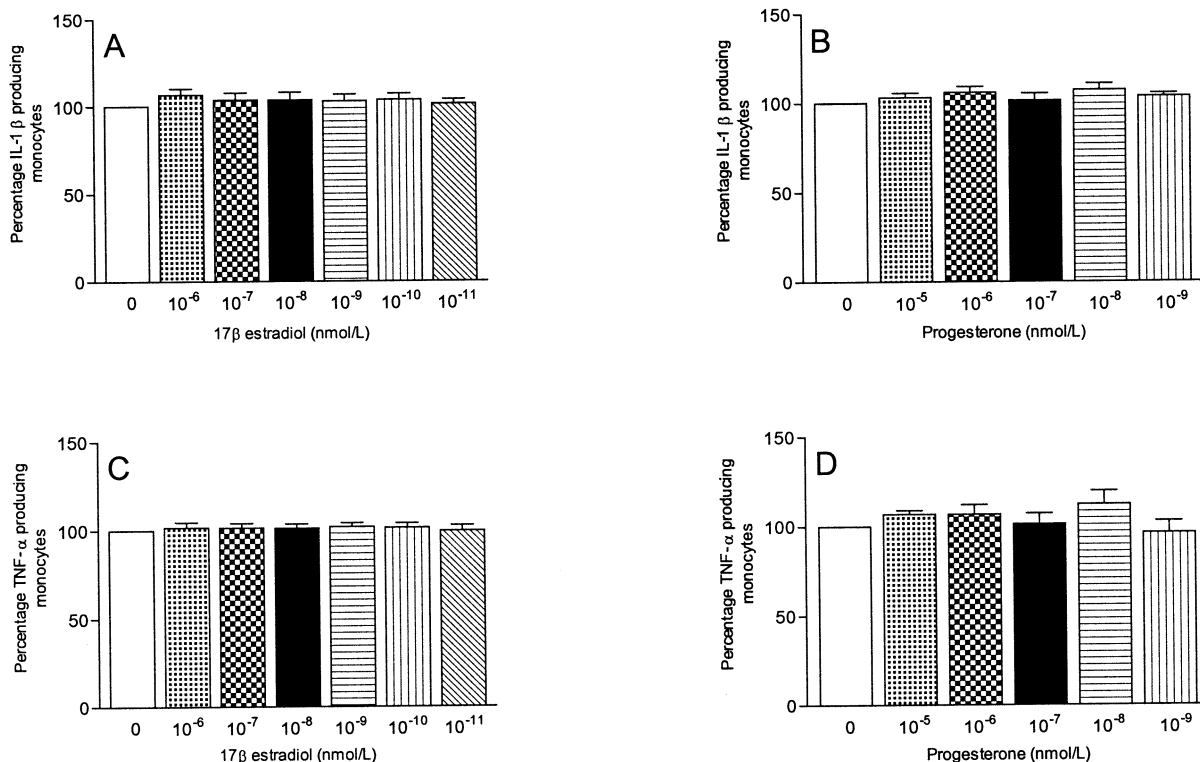
In line with our results, Rogers and Eastell (6) and Ralston et al. (7) could not demonstrate any effect of 17 β -estradiol on TNF- α production of LPS-stimulated peripheral blood mononuclear cells (PBMC) in postmenopausal women. On the other hand, Asai et al. (8) demonstrated decreased LPS-induced TNF- α production after incubation

of PBMC with 17 β -estradiol at both physiologic and supra-physiologic levels; Loy et al. (9) demonstrated that physiologic levels of both 17 β -estradiol and progesterone decreased TNF- α mRNA from peripheral blood monocytes.

Also, the ability of LPS-stimulated monocytes to produce IL-1 β was not influenced by 17 β -estradiol or progesterone. This is consistent with Stock et al. (10) and Rogers and Eastell (6) who described no statistically significant changes in IL-1 β production by postmenopausal stimulated monocytes after incubation with 17 β -estradiol (10, 6). In contrast, others have described an effect of 17 β -estradiol or progesterone on IL-1 β production of stimulated monocytes. Both the inhibition (11) and the stimulation (12, 13) by 17 β -estradiol or progesterone of stimulated monocytes that produce IL-1 β have been described.

Our in vivo studies were set up to investigate the effect of 17 β -estradiol and progesterone on monocyte cytokine production in vivo. In our study with OC users, we investigated

(A, B) Percentage of monocytes producing IL-1 β after in vitro incubation of whole blood of men, with different concentrations of (A) 17 β -estradiol and (B) progesterone. (C, D) Percentage of monocytes producing TNF- α after in vitro incubation of whole blood of men, with different concentrations of (C) 17 β -estradiol and (D) progesterone.



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the effect of synthetic hormones on monocyte production of cytokines. Therefore, pills were selected with low androgenicity. As expected, 17 β -estradiol concentrations were statistically significantly higher in the OC-free period as compared with day 17 of the OC period, indicating the effect of synthetic estrogens and progestogens on ovarian activity. Although, we cannot totally exclude follicular growth, dominant growth (>10 mm) was prevented as our patients used OCs with 30 or 35 μ g ethinylestradiol (14, 15).

Consistent with the results of Rogers and Eastell (16), in our study no difference in the percentage of monocytes producing TNF- α and IL-1 β was found between the OC-use and OC-free periods. Thus, we conclude that neither 17 β -estradiol nor synthetic hormones like ethinylestradiol, desogestrel, norgestimate, or gestodene influence the cytokine-productive capacity of LPS-stimulated monocytes in vivo. This is in line with our other in vivo study (experiment 1) in which we could not find any relation between 17 β -estradiol or progesterone and monocytes producing IL-1 β or TNF- α . The results from this in vivo study are in contrast with the results of Schwarz et al., who demonstrated a statistically significant, positive cor-

relation between the concentration of 17 β -estradiol in plasma and the release of TNF- α by LPS-stimulated monocytes in the luteal phase (17).

The discrepancies between our described results of in vitro and in vivo studies in humans could be due to the different methods we employed; for instance, varying results might have been produced by our use of different culture techniques (whole blood vs. peripheral blood mononuclear cells), isolation techniques, and stimuli, and monocytes from different types of subjects (women in various reproductive phases or men). Thus, it is difficult to compare the results from our studies. In the present study, to rule out as many confounding factors as possible, we measured intracellular cytokine production by in vitro activated monocytes in whole-blood preparations in both our in vitro and in vivo experiments.

Whole blood was used to avoid any activation of monocytes caused by isolation of these cells, and flow cytometry was used for analysis so that production of intracellular cytokines could be measured at a single-cell level (18, 19). Measurement of the percentage cytokine-positive monocytes correlates well with measurement of

cytokine levels in supernatant, such as used in the studies we have described (20).

In this way, we have tried to simulate the *in vivo* situation as best as possible, and we conclude from both our *in vivo* and *in vitro* studies that there is no effect of 17 β -estradiol and progesterone on the ability of monocytes to produce TNF- α and IL-1 β . Therefore, it is questionable whether the demonstrated increase in the percentage of monocytes producing TNF- α and IL-1 β during the luteal phase as compared with the follicular phase of our previous study was an effect of progesterone or 17 β -estradiol (2). The concept of a follicular factor modulating the immune response during the follicular phase, therefore, becomes more credible.

Other studies have shown differences in TNF- α and IL-1 β production that are independent of 17 β -estradiol and progesterone. Increased TNF- α production by LPS-stimulated monocytes in men as compared with women has been described (8, 17, Bouman et al., *in press*). Moreover, in rats, LPS-induced glomerular monocytes infiltration was inhibited only during the follicular phase, not in the luteal phase or after ovariectomy, with or without hormone supplementation (21, 22). This clearly suggests an inhibition of LPS-induced monocyte activity in the follicular phase. At present, we are working on the concept of monocyte inhibition during the follicular phase.

We conclude that we could not demonstrate any statistically significant effect of 17 β -estradiol or progesterone *in vitro* or *in vivo* on the cytokine-productive capacity of LPS-stimulated monocytes. Together with data from rat experiments (21, 22), this argues for the suppression of monocyte function during the follicular phase rather than an increase in monocyte function during the luteal phase. Such a suppression of the nonspecific immune response during the follicular phase may be necessary to prevent premature ovulation because ovulation is considered an inflammatory response. This, however, needs further investigation. Whether the variation in this nonspecific immune response within the ovarian cycle might be necessary for the physiologic preparation of the maternal immune system for potential implantation of the semiallogenic blastocyst also needs further investigation.

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