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Fluctuation of Peripheral Blood T, B, and NK Cells during a Menstrual Cycle of Normal Healthy Women

Sungki Lee,* Jeeyun Kim,* Byungwoo Jang,* Sungeun Hur,* Unsuk Jung,* Kihyun Kil,* Baegju Na,† Millina Lee,‡ Youngmin Choi,§ Atsushi Fukui,‖ Alice Gilman-Sachs,‖ and Joanne Y. Kwak-Kim‖,#

Cyclical hormonal changes during an ovarian cycle may affect immune responses, which is crucial for the embryonic implantation. We aim to investigate whether the levels and activity of T, B, and NK cells change during a menstrual cycle. Twenty-two normally cycling women were enrolled and peripheral blood was drawn serially during a menstrual cycle. Intracellular cytokine expression of CD3+ CD4+ and CD3+ CD8+ cells, and Th1/Th2 cytokine-producing T cell ratios were determined using flow cytometric analysis. NK cell cytotoxicity was measured by flow cytometric analysis at E:T ratios of 50:1, 25:1, and 12.5:1 and also using LU at 20%. Proportions (percentage) of CD3+ (p = 0.046) and CD3+ CD4+ (p = 0.002) T cells were increased in the follicular phase compared with the luteal phase. The levels of CD3+ CD56+ (p = 0.010) and CD3+ CD56dim+ (p = 0.012) NK cells and NK cytotoxicity at E:T ratio of 50:1, 25:1, and 12.5:1 and LU at 20% were significantly increased in the luteal phase compared with the follicular phase. Even though IL-10–producing CD3+ CD4+ T cells were significantly lower in the midluteal phase as compared with the early follicular phase, proportions of CD19+ B cells, CD3+ CD56+ NKT cells, Th1 cytokine-producing T cell subsets, and ratios of Th1/Th2 cytokine-producing T cells were not significantly changed during a menstrual cycle. We conclude that peripheral blood NK and T cell levels as well as NK cytotoxicity are changed during a menstrual cycle. Neuroendocrine regulation on immune responses is suggested during an ovarian cycle, which may be critical for embryonic implantation and pregnancy.

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Immunologic effectors closely interact with the endocrine system by initiating processes, which lead to the release of diverse mediators and cytokines that recruit endocrine and neural participation in immune response. In turn, the endocrine and neuronal system releases various hormones and neurotransmitters, which modulate immune responses (1). In fact, steroid hormones, especially gonadal sex steroids, have been reported to influence the immune system, and this interaction is mediated through the hypothalamic-pituitary-gonadal-thymic axis (1). Gonadotropin-releasing hormone (GnRH) and receptors are also produced locally by immune cells, suggesting an autocrine or paracrine role of GnRH in regulating immune functions (2).

There is clinical and laboratory evidence indicating how the endocrine and immune system affect each other. The prevalence of autoimmune disease is significantly higher in women as compared

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The online version of this article contains supplemental material.

Abbreviations used in this paper: E, early follicular; EL, early luteal; ER, estrogen receptor; GnRH, gonadotropin-releasing hormone; IVF, in vitro fertilization; KIR, killer Ig-like receptor; LF, late follicular; LL, late luteal; LU20, LU at 20%; MCD, menstrual cycle day; ML, midluteal; PR, progesterone receptor.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/S16.00 with men (3). More than 90% of Sjögren disease, scleroderma, and autoimmune thyroid disease develop in women (4). In postmenopausal women, numbers of CD4+ T and B cells are decreased, but CD8+ T cells are increased as compared with those of women in reproductive age (5). Estrogen replacement therapy reversed these immunological changes in women with surgically induced menopause (6). Hence, sex steroids seem to play a major role in homeostasis and activity of lymphocytes.

Estrogen has been reported to prevent B cell apoptosis, enhance survival and activation of autoreactive cells (7), and increase expression of CD4+ T cell chemokine receptors (8). Estrogen exerts immune regulation via estrogen receptors (ERs) on the lymphocytes, and receptors for estrogen, progesterone, androgen, and glucocorticoid are found in lymphoid organs and/or lymphocytes (9, 10). Compared with the ERs, nuclear progesterone receptor (PR) expression is restricted in peripheral blood NK cells, especially killer Ig-like receptor (KIR)+ cells (11). Through the PRs, progesterone induces apoptosis of NK cells and suppresses IL-12–induced IFN-γ production of KIR+ NK cells. Expression of PRs in T and B cells is insignificant and peripheral blood CD3+Bright, and uterine NK cells do not express PRs (11).

Gonadotropin and sex steroids are cyclically regulated during a menstrual cycle and even with diurnal rhythm. These cyclic changes were also seen in immune system. The levels of T and B cells have been reported to reach nadir between midnight and early morning and peak in the afternoon, although no significant circadian change was noted in NK cell levels (12). Total number of monocytes was reported to be significantly increased in the luteal phase as compared with follicular phase (12). The numbers of total CD2+, CD4+ T lymphocytes, and B and NK cells were positively correlated with luteinizing hormone and negatively with follicle stimulating hormone serum levels (5). However, the results are not consistent in terms of the levels and functional activity of lymphocytes (5, 12–14). These discrepancies may be
related to the study population, study design and sample size, and so on.

In women with recurrent pregnancy losses or multiple implantation failures, levels of peripheral blood NK cells and Th1/Th2 cytokine-producing cell ratios were significantly higher than those of normal fertile women (15, 16). Contrarily, T and NK cell levels and the Th1/Th2 cytokine ratio in peripheral blood of normal pregnant women are significantly decreased as compared with those of nonpregnant women (15, 17). Therefore, systemic immune regulation prior to and postconception is directly related to the reproductive outcome. Gonadal sex steroid and GnRH may be keenly related to systemic regulation of immune responses. However, the effect of ovarian cycle on immune effectors is remained largely elusive. In this study, we aim to investigate the levels of PBL subpopulations, NK cell cytotoxicity, and the levels of Th1 and Th2 cytokine-producing T lymphocytes during a menstrual cycle in healthy normal cycling women.

Materials and Methods

Study population

The study was designed as a prospective observational study. Study subjects were recruited at Konyang University Hospital (Daejeon, Korea). All had signed an informed consent before they entered the study. A total of 30 women were enrolled in the study; however, 8 dropped out as a result of emerging pregnancy, vaccination, inflammatory disease, and failure of matching sampling schedule. Finally, 22 women completed the study as scheduled. Age, obstetrical, and menstrual histories of study women are listed in Table I. All subjects were nonpregnant healthy women whose menstrual cycles had been regular. No one had active diseases, a history of spontaneous abortion, or autoimmune diseases. Eight women had a history of normal delivery and 14 had never been pregnant.

Laboratory

Peripheral blood was drawn consecutively for five times throughout the menstrual cycle between 8 and 10 AM. The menstrual cycle was divided into five phases: the early follicular (EF, menstrual cycle day [MCD] 2–4), the late follicular (LF, MCD 11–13), the early luteal (EL, MCD 16–18), the midluteal (ML, MCD 20–22), and the late luteal (LL, MCD 25–27) phases. A total of 7–10 mL blood was collected each time in a BDVacutainer with heparin (BD Biosciences, San Jose, CA). All specimens were immediately processed following blood sampling.

Immunophenotyping of the whole blood

Two-color direct immunofluorescence reagent kits (BD Simultest IMK-Lymphocyte; BD Biosciences) were used to enumerate percentages of the following mature human leukocyte subsets in erythroyte-lysed whole blood: CD3+ T, CD3+CD4+ (CD3+CD8+ T), CD3+CD8+ T, CD19+ B, NK (CD3+CD56+ and/or CD16+), and CD3+CD56+ NKT cells. IgG1 FITC and IgG2A PE were used for isotype controls.

Tubes containing 100 μL blood were incubated with each Ab at room temperature for 20 min. The BRCs were lysed with 2 mL erythrocyte-lysis solution (BD Biosciences). Tubes were incubated for 10 min at room temperature and washed twice with PBS (Life Technologies, Grand Island, NY) with 0.09% sodium azide (Sigma-Aldrich, St. Louis, MO). Cells were resuspended in the 0.5 mL 1% paraformaldehyde and stored at 4°C until flow cytometric analysis. The staining cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences). CellQuest Pro software (BD Biosciences) was used for data analysis.

Table I. Age, obstetrical, and menstrual histories of study women who have normal menstrual cycles (n = 22)

<table>
<thead>
<tr>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>31.1 ± 4.71</td>
</tr>
<tr>
<td>Gravidity</td>
<td>0.6 ± 0.84</td>
</tr>
<tr>
<td>Parity</td>
<td>0.6 ± 0.84</td>
</tr>
<tr>
<td>No. of spontaneous abortions</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Menstrual interval (d)</td>
<td>29.1 ± 1.84</td>
</tr>
</tbody>
</table>

Separation of PBMCs

PBMCs were isolated using Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density centrifugation with Leucosep tubes (Greiner Bio-One, Frickenhausen, Germany). After washing with HBSS (Life Technologies), the cells were adjusted to a final concentration of 5 × 10^6 cells/mL in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS (Life Technologies) and 1 mL antibiotic-antimycotic solution (100×; Life Technologies). Prepared PBMCs were stored at 4°C in the dark.

NK cell cytotoxicity study

NK cell cytotoxicity was determined at E:T cell ratios of 50:1, 25:1, and 12.5:1 (18). Target cells were prepared by culturing the cell line K562, a NK cell-sensitive human erythremocytic leukemia cell line, in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotic-antimycotic solution at 37°C in a 5% CO2 humidified incubator. A total of 5 × 10^5 K562 cells were washed twice in RPMI 1640 medium containing antibiotic-antimycotic solution and resuspended in 0.5 mL PKH2 dye (Sigma-Aldrich). In a separate 1.5-mL tube, 2 μL PKH2 dye (Sigma-Aldrich) was added to 0.5 mL PKH2 diluent. PKH2 dye solution was added to the K562 target cells and incubated for 2 min at room temperature. After incubation, 1 mL FBS was added to the cell suspension to stop the reaction. The cells were then washed three times with RPMI 1640 medium containing 10% FBS and 1% antibiotic-antimycotic solution, and the cell concentration was adjusted to 1 × 10^6 cells/mL. PBMCs and PKH2-labeled K562 cells were cocultured at E:T ratios of 50:1, 25:1, and 12.5:1 at 37°C in a 5% CO2 humidified incubator. After 2 h, 0.2 μL propidium iodide (Sigma-Aldrich) solution (10 mg/mL) was added, and the cells were vortexed. NK cell cytotoxicity was expressed as the percentage of dead K562 cells containing propidium iodide.

LUs were also calculated to compare NK cytotoxicities of each menstrual phase. LU is defined as the estimated number of effecter cells to kill a specific percentage of target cells. LU was calculated as follows: number of LU/10^6 effector cells = 10^6/[(TXP), T indicates number of target cells, and Xp is the E:T ratio required for target cell lysis of a particular percentage (19). LU at 20% (LU20) is computed from the dose-response cytotoxicity curves.

Th1 and Th2 intracellular cytokine study

To activate PBMCs, 1 mL 5 × 10^6 cells/mL of cell suspension was incubated with 25 ng/mL PMA (Sigma-Aldrich) and 1 μM ionomycin (Sigma-Aldrich) in polypropylene test tube for 16 h at 37°C in a 5% CO2 humidified incubator. A 0.2 μM GolgiPlug (protein transport inhibitor containing brefeldin A; BD Biosciences) was also applied at the beginning of culture incubation to inhibit cytokine secretion. Cell staining procedure was carried out according to the manufacturer’s instructions with the Cytofix/ Cytoperm kit (BD Biosciences). Briefly, PBMCs were washed in PBS with 0.09% sodium azide twice, followed by staining with the fluorochrome-conjugated mAbs specific for cell surface Ags and were incubated for 15 min at 4°C. Anti–CD3-PerCP and anti–CD8-FITC were used to identify T cell populations. The cell pellet was then fixed and permeabilized for 20 min using 250 μL Cytofix/Cytoperm solution (BD Biosciences). Next, PE-conjugated anti-cytokine Abs were used to stain PBMCs for 30 min. To detect intracellular cytokines, 0.2 μg mAbs for TNF-α, IFN-γ, and IL-10 were applied. Corresponding isotype controls were used for each Ab. After intracellular staining, the cells were washed twice with Perm/Wash buffer (BD Biosciences) and resuspended in 0.5 mL perm/wash buffer (16).

Statistical analysis

Statistical analysis of the data was performed using SAS Statistics (version 9.1; SAS Institute, Cary, NC). The distribution of the data was checked for normalcy by using a normal probability plot. The repeated measures ANOVA was applied to determine the statistical significances in variables repeatedly measured for five menstrual phases. Posthoc analysis was applied to determine the discriminating point for repeated measurement by using Duncan’s and Dunnett’s methods, which are sensitive to find patterns. In comparison of cytokine producing T cell subpopulations, paired t test was used.

Results

Table II shows that the percentages of CD3+ T and CD3+ CD4+ T (p = 0.002) cells are significantly changed during a menstrual cycle (EF and LF, EL, ML, and LL phases) of healthy women.
The percentages of TNF-\(
\alpha\) and IFN-\(\gamma\)-producing cells; percentages of TNF-\(\alpha\)-producing CD3+, CD3+CD4+, and CD3+CD8+ T cells were significantly lower than those of other phases. IFN-\(\gamma\)-producing CD3+, CD3+CD4+, and CD3+CD8+ T cells were also not significantly different between any two phases of a menstrual cycle. The level of IL-10–producing CD3+CD4+ T cells was decreased in the ML phase when compared with that of the EF phase. The levels of IL-10–positive CD3+ and CD3+CD8+ T cells were not significantly different between any two phases of menses.

The proportion of TNF-\(\alpha\)-producing CD3+CD4+ T cells was significantly higher than IFN-\(\gamma\)-producing CD3+CD4+ T cells throughout the cycle (\(p < 0.001\), each phase) (Table III). When comparing cytokine expression in CD3+CD4+ and CD3+CD8+ T cells, percentages of TNF-\(\alpha\)- and IL-10–producing cells were significantly higher in CD3+CD4+ T cells than CD3+CD8+ T cells in all five phases of the menstrual cycle (\(p < 0.001\) in TNF-\(\alpha\)-producing cells; \(p < 0.001\) for EF, LF, EL and LL phase; \(p < 0.001\) for phase in IL-10+) cells) (Fig. 2A, 2B). However, percentage of IFN-\(\gamma\)–producing CD3+CD4+ T cells were significantly lower than those of CD3+CD8+ T cells in all five menstrual phases (\(p < 0.001\) each) (Fig. 2C).

Th1/Th2 cell ratios in T cell subsets (CD3+, CD3+CD4+, and CD3+CD8+) were not significantly different during the menstrual cycle (Table IV). Th1/Th2 cell ratios in CD3+, CD3+CD4+, and CD3+CD8+ T cells did not show any significant changes between any two phases of ovarian cycle.

The ratios of TNF-\(\alpha\)/IL-10–producing cells (\(p = 0.054\) in ML phase; \(p < 0.05\) in EF and LL phases; \(p < 0.01\) in LF and EL phases) (Fig. 3A) and IFN-\(\gamma\)/IL-10–producing cells (\(p < 0.001\) in all five menstrual phases) (Fig. 3B) were significantly higher in CD3+CD8+ T cells than those of CD3+CD4+ T cells throughout the menstrual phases.

**Discussion**

In this study, we report statistically significant changes in percentages of peripheral blood CD3+ and CD3+CD4+ T cell levels throughout the menstrual cycle, which includes a decrease of those cell levels in the ML and LL phases as compared with EF phase. This finding may suggest a potential immunoregulatory
role of progesterone and/or estrogen on T cells. The potential down regulatory effect of sex steroids and/or pituitary gonadotropin has been suggested. In patients with Sheehan’s syndrome (n = 8), significantly higher pan T, Th, and cytotoxic T (CD2, CD4, and CD8) and B cells (CD19) were reported when compared with normal menstruating women (14).

In a PCR work, CD4⁺ T cells were reported to express relatively high levels of ERα mRNA as compared with ERβ, and B and NK cells were also reported to express ERα and ERβ (20, 21). It has been demonstrated that 17β-estradiol suppresses production of IL-2, IL-6, TNF-α, IL-1 receptor antagonist, and IL-1β from human peripheral blood T cells (22, 23). PRs are expressed on lymphocytes of pregnant women (24). Membrane PRα mRNA has been also detected in T cells, which is upregulated in the luteal phase of the menstrual cycle in CD8⁺ but not in CD4⁺ T lymphocytes (10). In addition, it has been shown that progesterone can suppress Th1 immune responses and enhance Th2 cytokine production in vitro (25). Therefore, both estrogen and progesterone have potential immune regulatory function through activation of their receptors.

T cell changes in this study are not consistent with the previous studies. Coulam et al. (26) reported no variation in the percentage of Leu-2, Leu-3, or Leu-4 cells during the menstrual cycle (n = 5) or oral contraceptive cycle (n = 5). Later on, Lopez-Karpovitchs et al. (14) reported that normal women (n = 6) showed little nonstatistically different changes along the menstrual cycle in peripheral blood cell parameters. Discrepancies in these studies as compared with ours can be explained by 1) number of study population, 2) different cell markers, and 3) different blood sampling time.

In this study, NK cell levels and cytotoxicity are statistically significantly different along the five menstrual phases. The major
changes were NK cell levels between the EF and the ML and LL phases. In addition, NK cytotoxicity was significantly increased during the ML phase as compared with EF and LF phases both in

Table III. Percentage of CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ cells expressing intracellular Th1 (TNF-α and IFN-γ) and Th2 (IL-10) cytokines during a menstrual cycle of normal healthy women (n = 22) by flow cytometric analysis

<table>
<thead>
<tr>
<th>Phase of Menstrual Cycle</th>
<th>EF</th>
<th>LF</th>
<th>EL</th>
<th>ML</th>
<th>LL</th>
<th>p Value among Five Phases</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>58.84 ± 9.97</td>
<td>58.91 ± 8.06</td>
<td>59.24 ± 9.10</td>
<td>59.00 ± 8.36</td>
<td>60.76 ± 8.75</td>
<td>0.785</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>29.96 ± 9.16</td>
<td>31.15 ± 7.29</td>
<td>30.30 ± 9.67</td>
<td>29.20 ± 9.13</td>
<td>31.38 ± 8.88</td>
<td>0.749</td>
</tr>
<tr>
<td>IL-10</td>
<td>2.98 ± 0.87</td>
<td>2.53 ± 0.66</td>
<td>2.77 ± 0.87</td>
<td>2.61 ± 0.84</td>
<td>2.70 ± 0.58</td>
<td>0.338</td>
</tr>
</tbody>
</table>

% CD3⁺ T cells

<table>
<thead>
<tr>
<th>Phase of Menstrual Cycle</th>
<th>EF</th>
<th>LF</th>
<th>EL</th>
<th>ML</th>
<th>LL</th>
<th>p Value among Five Phases</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>66.26 ± 12.84⁺</td>
<td>67.80 ± 8.98⁺</td>
<td>67.44 ± 11.75⁺</td>
<td>66.94 ± 12.20⁺</td>
<td>70.06 ± 12.43⁺</td>
<td>0.243</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>26.38 ± 9.53</td>
<td>27.20 ± 8.53</td>
<td>26.60 ± 10.51</td>
<td>24.32 ± 10.37</td>
<td>26.36 ± 9.93</td>
<td>0.299</td>
</tr>
<tr>
<td>IL-10</td>
<td>4.46 ± 2.54ᵇ</td>
<td>3.52 ± 1.49</td>
<td>3.56 ± 2.09</td>
<td>2.96 ± 1.05</td>
<td>3.86 ± 1.76</td>
<td>0.066</td>
</tr>
</tbody>
</table>

% CD3⁺CD4⁺ T cells

<table>
<thead>
<tr>
<th>Phase of Menstrual Cycle</th>
<th>EF</th>
<th>LF</th>
<th>EL</th>
<th>ML</th>
<th>LL</th>
<th>p Value among Five Phases</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>44.75 ± 11.98</td>
<td>44.16 ± 10.26</td>
<td>44.64 ± 11.16</td>
<td>43.38 ± 11.81</td>
<td>45.44 ± 10.45</td>
<td>0.743</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>41.51 ± 11.71</td>
<td>41.78 ± 10.72</td>
<td>41.48 ± 11.50</td>
<td>39.39 ± 9.97</td>
<td>41.73 ± 11.18</td>
<td>0.708</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.84 ± 1.11</td>
<td>1.65 ± 1.00</td>
<td>1.65 ± 1.06</td>
<td>1.69 ± 1.38</td>
<td>1.86 ± 1.33</td>
<td>0.462</td>
</tr>
</tbody>
</table>

% CD3⁺CD8⁺ T cells

Data (%) are expressed as mean ± SD.

⁺Significantly different between TNF-α and IFN-γ-producing CD3⁺CD4⁺ T cells at each menstrual phase (p < 0.001).

ᵇSignificantly different between two T cell subpopulations producing TNF-α, IFN-γ, or IL-10 in each menstrual phase are plotted with asterisks (⁎⁎⁎p < 0.01; ⁎⁎⁎⁎p < 0.001).

LUs and E:T ratios. Our data are consistent with the previous study that NK cell levels are significantly increased during the luteal phase without any changes in pattern of cytokine production (27). Contradictory results have been also reported. Yovel et al. (28) reported no changes in NK cell concentration during a menstrual cycle except periovulatory increase in the number of NK cells per milliliter of blood. Because we studied proportion (percentage) of NK cells in lymphocyte population by flow cytometry, a direct comparison of our data with that of Yovel et al. (18) cannot be made. Previously, we have reported lack of correlation between peripheral blood NK cell cytotoxicity and the percentages or absolute counts of CD56⁺CD16⁻, CD56⁺CD16⁺, or CD3⁺CD56⁺ lymphocyte subsets. In addition, we and others found percentage of peripheral blood NK cells were significantly increased in women with recurrent pregnancy losses or implantation failures (15, 29). Therefore, in this study, we decided to measure proportion of NK cells only instead of actual NK cell count per milliliter. Northern et al. (12) reported no changes in NK cell levels between days 6 and 22. As we sampled two times during follicular phase (EF, CD 2–4; LF, CD 11–13), our data cannot be directly compared with that of Northern et al. (12). However, it is noteworthy that there are no significant differences in NK cell levels between LF and ML phases in our study.

Studies are inconsistent in regard to NK activity during a menstrual cycle. Souza et al. (30) reported increased NK activity in follicular phase as compared with luteal phase. Contrary to this, Sulke et al. (31) reported a significantly decreased NK cytotoxicity during periovulatory period as compared with normal male volunteers. This change was reported not to be apparent in women taking oral contraception. Others reported no changes in NK activity during a menstrual cycle (28).

Our results of significant changes in NK cell levels and cytotoxicity during the ML phase may be explained as the effect of increased levels of progesterone and 17β-estradiol. In animal studies, estrous cycle modulates adrenergic suppression of NK activity (32). Both of ERα and ERβ are present in murine NK cells and pregnancy level of estrogen-suppressed NK cytotoxicity via ERβ or other receptors (21). In contrast, estrogen depletion by ovariectomy lowered NK activity in rhesus monkeys (33). These studies show that estrogen regulates NK cytotoxicity in bimodal pattern by concentration (i.e., stimulatory at low estrogen level but inhibitory at pregnancy level). NK cytotoxicity has been known to be upregulated by IFN-γ, adrenocorticotropic hormone, β-endorphin, and prolactin and downregulated by glucocorticoids, lymphocyte
immunotherapy, and i.v. IgG (30, 34). Progesterone was also reported not to influence on NK activity (30). However, recently, PR was identified in KIR+ NK cells (11), but function of PR of NK cells still remained to be explored. Interestingly, Shakhar et al. (32) suggested that β-adrenergic stimulation acted as a suppressor of NK cytotoxicity through β-adrenergic receptors, which were increased during the luteal phase. These findings suggest that complicated neuroendocrine regulatory system(s) are involved in regulation of NK cytotoxicity.

Our observation of significant increase of NK cell level in the ML and LL phases compared with the EF phase may be connected to the immunobiology of implantation. Uterine NK cells are dominant lymphocytes and dramatically increase during the implantation window, late secretory-phase endometrium, and the decidua during pregnancy. Exact mechanism of NK cell surge in the endometrium during this period remains to be elucidated. Ovarian steroid hormones seem to be deeply involved in recruitment of uterine NK precursors (35). Estrogen promotes CD56++ NK cell homing to the uterus. Progesterone also helps CD56++ NK cells entering to the uterus and proliferation of uterine NK cells through stimulation of IL-15, vascular endothelial growth factor, prolactin, and other growth factors from endometrial stromal cells (36). Trafficking of peripheral blood NK cells to the uterus is proposed as one of putative mechanism of uterine NK cell increase. Trophoblasts and endometrial stromal cells express chemokines and adhesion molecules, which are possibly involved in the recruitment of NK cells (37). Furthermore, there is a report that CD56+CD16- cells can be transformed to CD56+CD16+ NK cells by TGF-β in vitro, which is produced locally in the secretory endometrial and decidua tissues (38, 39). Thus, TGF-β may convert CD56+CD16- NK cells to CD56+CD16+ cells in the uterus. We suggest that the increase of peripheral blood NK cells in the luteal phase may contribute to recruitment of uterine NK cells from peripheral blood.

The mechanism controlling the cytokine production in T cells during the menstrual cycle remains to be elucidated. Whitleac et al. (40) described that low concentration of estrogen and prolactin induced Th1 responses, but high levels of estrogen such as the concentration in pregnancy, progesterone, and testosterone promote to develop a Th2 response. In our study, TNF-α and IFN-γ T cell populations were not fluctuated during the menstrual cycle. However, there were significant changes between the EF and ML phases in terms of the level of IL-10-producing CD3+CD4+ T cells. No such prominent changes were found in the ratio of Th1/Th2 cytokine-producing CD3+CD4+ T or CD3+CD8+ T cells. Because there are no other studies measuring cytokine producing cells during a menstrual cycle, we cannot compare our data with others. However, it is noteworthy that the previous report of comparing cytokine profiles between the follicular and luteal phases did not demonstrate any differences in production of IL-4, IL-2, and IL-10 (13). Progesterone is known to suppress Th1 response and enhance IL-10-producing Th2 cells (25). At concentrations in the placenta, progesterone-suppressed immune functions of T cells by a nongenomic mechanism, which is the blocking of K+ channel, Ca2+ signaling and NF of activated T cells driven gene expression (41).

Previously, we have reported that women with a history of recurrent spontaneous abortions or multiple implantation failures demonstrated significantly elevated peripheral blood NK cell levels, NK cytotoxicity, and Th1/Th2 cell ratios as compared with those of normal fertile women (16, 29, 42). In a study conducted in women undergoing in vitro fertilization (IVF) and embryo transfer, the Th1/Th2 ratios of IFN-γ/IL-4 and TNF-α/IL-4 measured on the day of oocyte retrieval were significantly lower than the ratios before controlled ovarian stimulation, especially in women with successful

### Table IV. Ratio of Th1 (TNF-α and IFN-γ)/Th2 (IL-10) cytokine producing T cells or subpopulations during a menstrual cycle of normal healthy women (n = 22) by flow cytometric analysis

<table>
<thead>
<tr>
<th>Phase of Menstrual Cycle</th>
<th>EF</th>
<th>LF</th>
<th>EL</th>
<th>ML</th>
<th>LL</th>
<th>p Value among Five Phases</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD3+ T cells</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TNF-α/IL-10</td>
<td>22.83 ± 8.88</td>
<td>24.84 ± 7.31</td>
<td>23.50 ± 8.14</td>
<td>24.81 ± 8.43</td>
<td>24.06 ± 9.03</td>
<td>0.755</td>
</tr>
<tr>
<td>IFN-γ/IL-10</td>
<td>11.19 ± 5.57</td>
<td>13.20 ± 5.04</td>
<td>11.83 ± 4.84</td>
<td>12.49 ± 5.71</td>
<td>12.50 ± 5.78</td>
<td>0.731</td>
</tr>
<tr>
<td><strong>CD3+CD4+ T cells</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>TNF-α/IL-10</td>
<td>20.96 ± 14.32</td>
<td>23.22 ± 11.58</td>
<td>23.71 ± 11.85</td>
<td>25.58 ± 9.86</td>
<td>23.12 ± 15.57</td>
<td>0.735</td>
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<tr>
<td>IFN-γ/IL-10</td>
<td>8.06 ± 5.53</td>
<td>8.68 ± 3.62</td>
<td>9.36 ± 6.63</td>
<td>9.39 ± 5.21</td>
<td>8.86 ± 8.69</td>
<td>0.892</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD.
IVF and embryo transfer (43). The ratios were increased in women with failed IVF. In our study, Th1/Th2 ratios are stable throughout the ovarian cycle even with significantly fluctuating hormonal values. Further study is needed to elucidate this question.

This study is a prospective longitudinal investigation of immune effectors and their functional activity in relation to five phases of the menstrual cycle. A majority of previous investigations were designed as a cross-sectional study, and some studies did not have the same time for blood drawing or the phase in a menstrual cycle. We report significant changes in immune effectors during the menstrual cycle. However, the patterns of changes are different from hormonal cycles and actual magnitude of changes is relatively small. This observation suggests the presence of multiple neuroendocrine regulations on immune effectors during ovarian cycle. Further investigation is needed to define the interaction of hormones and their receptors on the immune competent cells and their signaling pathways as well as cross-talk between neuroendocrine and immune system.

Disclosures

The authors have no financial conflicts of interest.

References