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ORIGINAL RESEARCH ARTICLE

The cytokine profile of menstrual blood

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Abstract

Introduction: The menstrual cycle is regulated by a complex interplay between endometrial epithelial cells, endothelial cells, immune cells, and sex hormones. To communicate, cells secrete cytokines that have multiple and diverse effects on recipient cells. Knowledge of how these cells interact in the uterus is insufficient. Menstrual blood is easily accessible and provides a source to study menstrual cycle physiology. This study aimed to determine the cytokine profile in menstrual blood plasma and investigate the differences in cytokine profiles between menstrual and peripheral blood plasma. Several previous studies indicate an improved chance of embryo implantation after endometrial scratching. Consequently, our secondary aim was to compare the menstrual blood cytokine profile before and after luteal phase endometrial scratching.

Material and methods: Nineteen healthy donors collected menstrual blood for the first 24 hours of menstruation in two sequential cycles. Matched peripheral blood was taken at the same time. An endometrial biopsy was performed at cycle day 7-9 post ovulation in between the two collection times. A Luminex multiplex assay was performed in one batch analyzing a predetermined group of cytokines in plasma.

Results: Peripheral blood plasma and menstrual blood plasma showed substantial significant differences in cytokine profile. In menstrual blood plasma, C5/C5a, interleukin-6 (IL-6), IL-1β, and CXCL8 were detected in high concentrations, whereas IL-2, IL-12p70, XCL1/Lymphotactin, and interferon-γ were low. The most pronounced median differences between menstrual and peripheral blood plasma were found for IL-6, IL-1β, and CXCL8. The cytokine profiles of menstrual blood plasma were similar between the individual donors and did not differ over two subsequent cycles. None of the cytokines analyzed in menstrual blood plasma differed significantly before or after luteal phase endometrial scratching ($P < .01$).

Abbreviations: C5/C5a, complement component 5; CCL3, chemokine (C-C motif) ligand 3; CXCL, chemokine (C-X-C motif) ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFNγ, interferon-γ; IL, interleukin; NK cell, natural killer cell; TNF, tumor necrosis factor (previously known as TNF-α); VEGF, vascular endothelial growth factor; XCL1/Lymphotactin, chemokine (C motif) ligand 1.

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Conclusions: Our results demonstrate that the menstrual blood cytokine profile is distinctly different from peripheral blood plasma and that the inter-individual difference in menstrual blood cytokine profile in healthy donors is limited and stable over time. The small injury caused by an endometrial biopsy does not change the cytokine profile in the subsequent menstrual cycle. Our study provides new insights into menstrual cycle physiology.

KEYWORDS

chemokine, cytokine, endometrial scratching, interleukin, menstrual blood, multiplex assay

1 | INTRODUCTION

Menstruation is orchestrated by hormonal signaling leading to a local, ischemic, and immunological reaction within the uterus that leads to endometrial shedding.[1,2] Despite this knowledge, no detailed descriptions of the cytokine profile of menstrual blood in health and disease exist. No studies have described a broad cytokine profile of menstrual blood and previous reports have focused on a limited number of inflammatory markers.[3,4] Cytokines (chemokines, interferons, interleukins, tumor necrosis factor, and lymphokines) are small proteins especially important for cell signaling in immune responses. Cytokines may be released by immune cells, endothelial cells, and stromal cells. After binding to their respective ligands, cytokines mostly exert their effects on immune cells in an autocrine, paracrine, or endocrine manner.[5] Sex hormones coordinate the menstrual cycle and are also known to induce the secretion of a number of cytokines present in the uterine endometrium that are required for angiogenesis, proliferation of natural killer (NK) and T cells, decidualization, and blastocyst implantation.[6-10] As menstruation is marked by local inflammatory processes and cytokines are pivotal for these processes, it is important to understand the difference between cytokine release in menstrual blood compared with peripheral blood. The local immunological milieu of the uterus may be influenced by various factors such as bacterial and viral infection, presence of semen, pregnancy, or iatrogenic mechanical wounding (eg endometrial biopsy, hysteroscopy, or hydrosonography).[11-14] Accordingly, the immunological milieu could differ between different cycles in the same women. Furthermore, and although still debated, endometrial scratching (through endometrial biopsy or extraction of an intrauterine device) could potentially provoke an immunological response favoring implantation.[15-17] Gnainsky et al[18] show that a local injury to the endometrium induces an inflammatory response that is characterized by a domination of pro-inflammatory cytokines and chemokines secreted by the endometrial stromal cells. Further, in vitro these pro-inflammatory cytokines attract monocytes and enhance the differentiation into dendritic cell-like cells. Endometrial stromal and endothelial cells respond to the dendritic cell-like cells by expressing the specific chemoattractants and adhesion molecules that are necessary in the interaction between the trophoblast and endometrium.

Key message

This study was designed to describe cytokine expression in menstrual blood. We detected a distinct menstrual blood cytokine profile, similar between individuals and different to peripheral blood. Endometrial scratching did not alter the menstrual blood cytokine profile.

The primary aim of this study was to investigate the difference between the menstrual and peripheral blood cytokine profiles. Second, we aimed to compare the menstrual blood cytokine profile before and after luteal phase endometrial scratching.

2 | MATERIAL AND METHODS

2.1 | Participants and methods

Study participants were recruited through advertisement at a web page for research volunteers. The inclusion criteria were: women 18-35 years of age, body mass index 19-25 kg/m^2, nulligravidae, with regular menstrual cycles. The exclusion criteria were previous gynecological surgery, continuous medications, hormonal contraception, chronic disease, use of intrauterine device at any time, and smoking. All women underwent a general physical examination, pelvic examination including transvaginal ultrasound, cervical smear test, and *Chlamydia* sampling. Vaginitis and bacterial vaginosis were ruled out by the clinical examination. Hormone analyses (sex hormone binding globulin, testosterone, estradiol, progesterone, prolactin, thyroid-stimulating hormone, and 17-hydroxyprogesterone) were taken if indicated. Four of the included women had hormonal analysis taken based on a multifollicular appearance of the ovaries when examined with transvaginal ultrasound. They all had normal hormone levels. None of the included women had any symptoms of endometriosis or polyps such as dysmenorrhea, dyspareunia or irregular bleedings. Further, no women had a history of infertility. A total of twenty-eight women entered the study. Nine women were

excluded because of screening failure or withdrawal of informed consent. Nineteen healthy women with median age of 28 years (range 19-32 years) and median body mass index 22.2 kg/m^2 (range 20.3-23.6 kg/m^2) continued in the study (Table 1). They were asked to collect menstrual blood using a menstrual cup (Lunette™) in two consecutive cycles. The menstrual blood was transferred by the study participants to a 50-mL Falcon tube prefilled with 5 mL cell medium containing heparin, penicillin/streptomycin, gentamicin, and gluconazole as previously described.[19] Menstrual blood samples were kept refrigerated until processed and frozen down.

The women provided the menstrual blood sample from the first 24 hours of the cycle, to obtain samples from the start of endometrial shedding. Peripheral blood samples were taken as controls on the same day as menstrual blood was processed. Menstrual blood volumes collected varied between 5 and 40 mL after adjustment of dilution with prefilled medium (see Section 2). Ovulation was detected by a urinary immune-based ovulation test (Clearblue®; SDP Swiss Precision Diagnostics GmbH). Endometrial scratching was performed 7-9 days after the luteinizing hormone surge by endometrial biopsy, using an aspiration biopsy cannula (Endorette™; Medscand®). A second menstrual blood sample was collected at first menstruation after endometrial sampling.

Peripheral and menstrual blood samples were centrifuged at 500 ***g*** for 5 minutes, and the plasma phases were collected and frozen in −80°C until use. Out of the 19 women, 11 donated menstrual and peripheral blood at both time-points (Table 1). Peripheral blood from a male external control was used as assay control. This assay control sample was divided into three aliquots. The first aliquot was treated as a regular peripheral blood sample where plasma was collected (see Figure 1E, control 1). The second and third aliquots, were treated as menstrual blood and diluted in collection medium but kept refrigerated (Figure 1E, control 2) or diluted in collection medium, but kept at room temperature for 24 hours (Figure 1E, control 3), respectively.

A panel of 20 cytokines, chemokines, and growth factors, was carefully selected. Menstrual blood and peripheral blood samples were analyzed for amphiregulin, complement component 5/5a (C5/C5a), chemokine (C-C motif) ligand 3/macrophage inflammatory protein 1α (CCL3/MIP-1α), chemokine (C motif) ligand 1/Lymphotactin (XCL1/Lymphotactin), chemokine (C-X-C motif) ligand 10 (CXCL10), chemokine (C-X-C motif) ligand 8 (CXCL8/IL-8), granulocyte-macrophage colony-stimulating factor (GM-CSF), granzyme A, granzyme B, interferon-γ (IFN-γ), tumor necrosis factor (TNF), interleukin-1β (IL-1β), IL-10, IL-12p70, IL-15, IL-18, IL-2, IL-6, osteopontin, and vascular endothelial growth factor (VEGF) using the human Magnetic Luminex assay (R&D Systems). These were selected based on previous studies indicating effects on immune cells present in the tissue, or production by immune cells in the tissue, including NK cells: osteopontin,[20] VEGF,[21] CXCL10,[22] CCL3, GM-CSF, IL-1β, IL-2, IL-6, IL-8, IL-12, IL-15, IL-18, XCL1, TNF, IFN-γ,[23-26] perforin and granzymes,[27] C5/C5a was included because of availability and potential relevance due to its immune activity. The experimental procedure was performed according to the manufacturer's instructions apart from an altered sample dilution ratio, for which we corrected in downstream analysis.

A Luminex MAGPIX instrument (Merck Millipore) was used to analyze the concentration of cytokines in plasma. The measured concentrations were corrected for both the intra-experimental dilution and for the menstrual blood sampling dilution of collection medium.

Values below or above the limit of detection were replaced with the respective limit of detection (highest or lowest standard concentration), values within the detection range were used after correction for dilution, as described above (see Supplementary material, Figure S1).

TABLE 1 Characterization of study participants

Clinical parameters	Healthy study participants (n = 19)
Age (years), median (range)	28 (19-32)
BMI (kg/m^2), median (range)	22.2 (20.3-23.6)
Menstruation duration (days), median (range)	4 (3-7)
Menstrual cycle length (days), median (range)	29 (25-32)
Sampled longitudinally	11

2.2 | Statistical analyses

SPSS Statistics 25 was used for comparison of median cytokine concentrations. Wilcoxon signed rank test was used to test differences in median cytokine concentrations between menstrual and peripheral blood plasma and between menstrual blood plasma at cycles 1 and 2. The significance level was set to $P < .05$. Correlations of cytokine concentrations were tested using Spearman's rank correlation test in GraphPad Prism. Because of the large number of correlation comparisons, it was decided to reject the null hypothesis only for correlations with a P-value of $<.01$.

2.3 | Ethical approval

Ethical approval was granted by the Regional Ethics committee in Stockholm, Sweden (DNR 2013/1324-31/2 ; date of approval: 2013-10-14 and 2014/1996-32; date of approval: 2014-11-24). All participants provided written informed consent to participate in the study.

3 | RESULTS

3.1 | Description of menstrual blood cytokine profile

To characterize the menstrual blood cytokine profile, we first measured the concentrations for each studied cytokine for

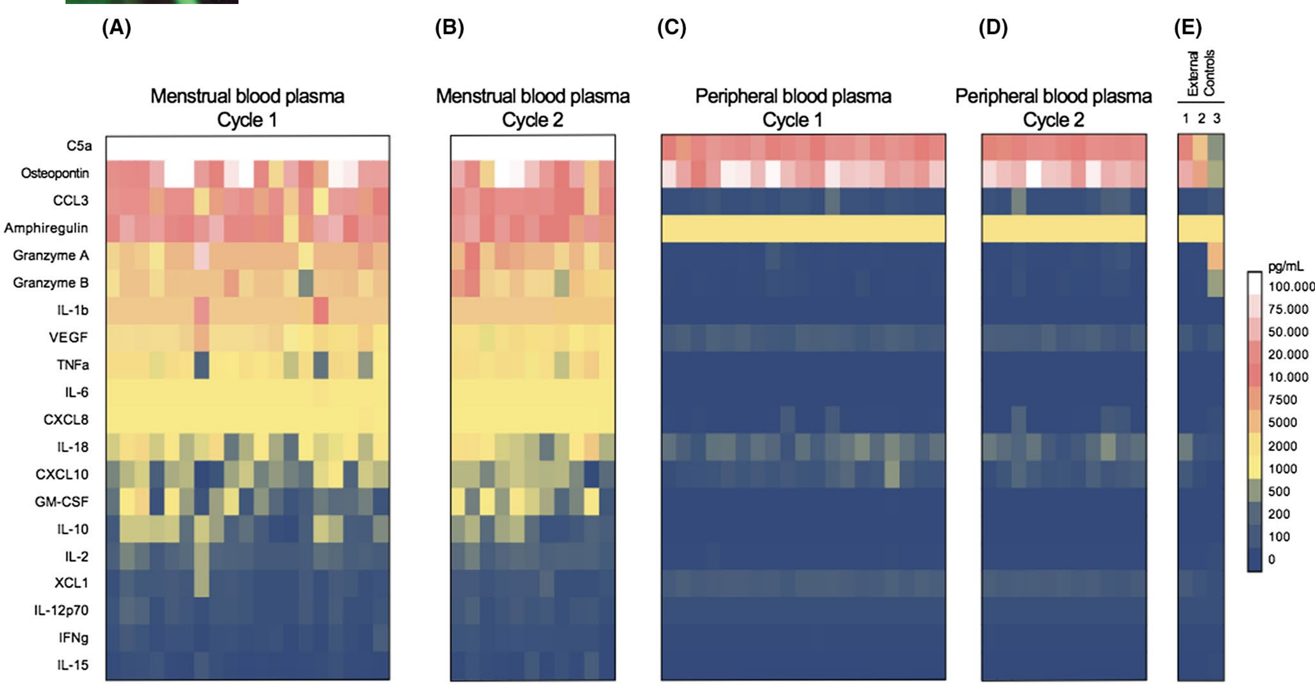

FIGURE 1 Heat map displaying cytokine concentrations measured in menstrual blood plasma in cycle one (A) before and cycle two (B) after endometrial scratching. Peripheral blood from cycle one (C) and cycle two (D). External controls (E) are plasma-derived from peripheral blood (control 1), peripheral blood collected in collection tube with collection medium kept refrigerated (control 2) or left at room temperature (control 3). C5/C5a, complement component 5; CCL3, chemokine (C-C motif) ligand 3; CXCL, chemokine (C-X-C motif) ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; XCL1, chemokine (C motif) ligand 1 [Color figure can be viewed at wileyonlinelibrary.com]

each donor in cycle one, which we summarized in a heat map (Figure 1A). The cytokine profiles of menstrual blood plasma were similar among the individual donors, displaying a similar pattern in menstrual blood. C5/C5a, IL-6, IL-1β, and CXCL8 were found in the highest concentrations, whereas IL-2, IL-12p70, XCL1/Lymphotactin, and IFN-γ where found in the lowest concentrations.

To validate that the measured cytokines were of endometrial origin we compared the menstrual samples with the external controls (Figure 1A,E). Only Granzyme A and B appeared to be impacted by storage and or dilution media, whereas all other cytokines showed similar levels between menstrual blood plasma and untreated peripheral blood plasma.

3.2 | Difference between menstrual and peripheral blood cytokine profiles

Next, we compared the cytokine profiles obtained from menstrual blood and peripheral blood. We used the median concentrations of each cytokine (Figure 1A,C). All the analyzed cytokines were significantly different ($P < .01$ for all) except osteopontin ($P = .72$). The most pronounced differences were demonstrated for IL-6, IL-1β, and CXCL8. The overall cytokine profile of menstrual blood differed substantially from peripheral blood. Notably, most cytokines showed higher concentrations in menstrual blood than peripheral blood (Figure 1A-D; see Supplementary material, Figure S2).

We also used an external peripheral blood plasma sample as reference. This was divided into three fractions and treated differently as described in the Material and methods section. The cytokine profiles of the first and second control samples (Figure 1E, control 1 and 2) were very similar to the cytokine profiles of the rest of the peripheral blood plasma samples (Figure 1C,D). The third control sample (Figure 1E, control 3), however, showed a similarity to the menstrual blood plasma samples with regard to granzyme A and B, and osteopontin. This indicates that the handling of the samples did not affect most cytokines.

Taken together, this illustrates that menstrual blood plasma has a cytokine profile that is distinctive from the one in peripheral blood plasma.

3.3 | Menstrual blood cytokine profile before and after endometrial scratching

To test if luteal phase endometrial scratching contributes to a difference in menstrual blood cytokine profile, we compared menstrual blood before and after an endometrial biopsy (Figure 1A,B). However, our results show that peripheral blood cytokines were stable throughout this time (Figure 1C,D). None of the cytokines analyzed in menstrual blood differed significantly before or after luteal

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phase endometrial scratching (*P* = .173 to *P* >.99) (Figure 2). Thus, in this setting we found no evidence that luteal phase endometrial scratching evokes a change in the subsequently analyzed menstrual blood cytokine profile.

3.4 | Correlation of cytokine concentrations in menstrual and peripheral blood plasma

To explore if the concentrations of certain cytokines in menstrual blood were associated, we performed a correlation analysis of each cytokine to all other cytokines analyzed (Figure 3A). Altogether, 14 significant ($P < .01$) positive correlations were identified. The most frequent correlations were found for both IFN-γ and IL-10. IFN-γ correlated with TNF, CCL3, CXCL10, and IL-12p70 and IL-10 with XCL1/Lymphotactin, amphiregulin, IL-2, and IL-15. In peripheral blood plasma (Figure 3B), a total of six significant ($P < .01$) positive correlations were noted. None of the significant correlations found for cytokines in peripheral blood plasma were similar to those identified to be significant in menstrual blood. Both GM-CSF and C5/C5a correlated with two cytokines, TNF, IL-2 and CCL3, IL-18, respectively. Taken together, the analyses revealed more cytokine correlations in menstrual blood plasma, and these correlations were not found in peripheral blood plasma. This further implies that the cytokine profile in menstrual blood plasma is distinctly different from peripheral blood.

4 | DISCUSSION

This study was designed to provide a broad overview of the cytokine profile in menstrual blood plasma of healthy individuals. Further, we compared the cytokine profile between menstrual and peripheral blood plasma and investigated the difference in menstrual blood plasma before and after an intervention to provoke an inflammatory response (luteal phase endometrial scratching).

Previous studies have investigated cytokines in menstrual blood using enzyme-linked immunosorbent assay on aspirate from the cervical canal, where TNF and VEGF were evaluated in healthy individuals who were serving as controls in a study of endometriosis.[4] By collection of menstrual blood aspirated from the internal cervical ostium, Tortorella et al studied IL-6, IL-1β, and TNF in healthy controls for a study of chronic endometritis.[3] Although the concentrations were generally low (<100 pg/mL), unfortunately neither study reported the absolute concentrations of the cytokines in a way that would make them directly comparable to our study. Hosseini et al[28,29] compare immune cells in menstrual blood with peripheral blood immune cells. However, these studies have focused on cells and intracellular cytokines and not free extracellular cytokines as in our study.

The inter-individual differences in cytokine profile of menstrual blood between donors were strikingly low, meaning that all participants demonstrated a similar pattern in the concentrations of menstrual blood plasma cytokines. The cytokines found in the highest concentrations—C5/C5a, IL-6, IL-1β, and CXCL8—are all pro-inflammatory.[30]

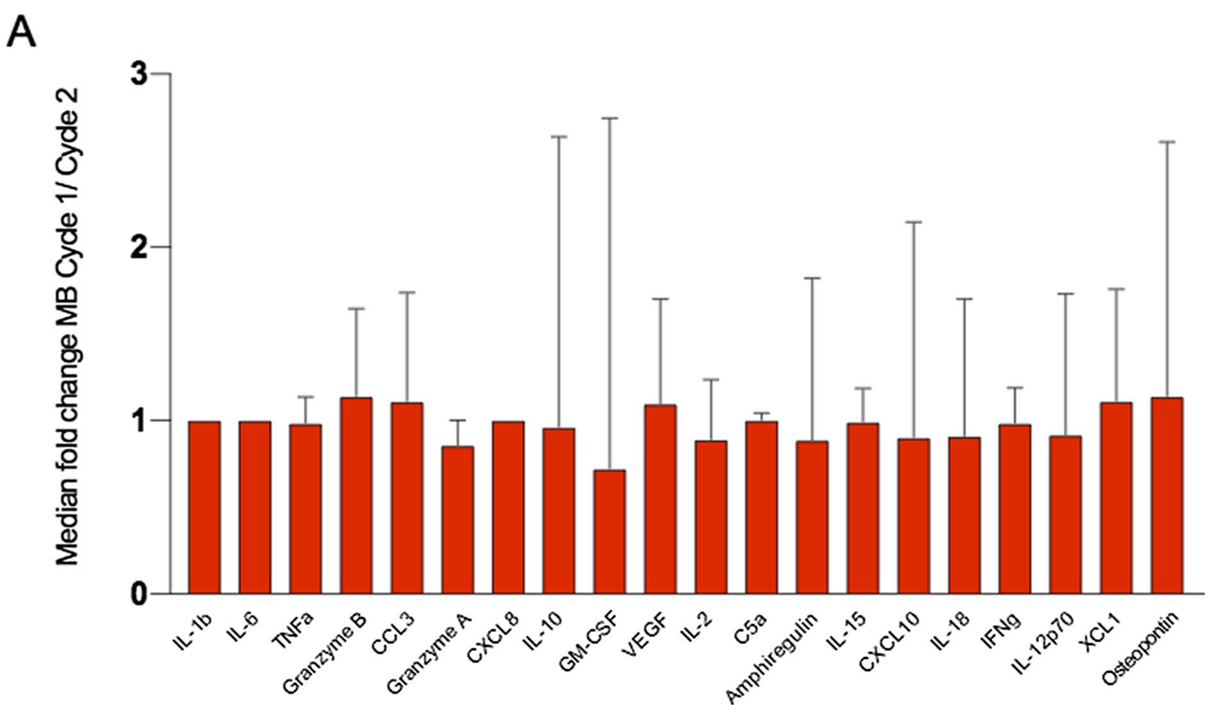

FIGURE 2 Fold change of indicated cytokines in menstrual blood plasma from cycle one compared with cycle two. Displayed are median values with range, only data from paired samples were included (n = 11). C5a, complement component 5; CCL3, chemokine (C-C motif) ligand 3; CXCL, chemokine (C-X-C motif) ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; XCL1, chemokine (C motif) ligand 1 [Color figure can be viewed at wileyonlinelibrary.com]

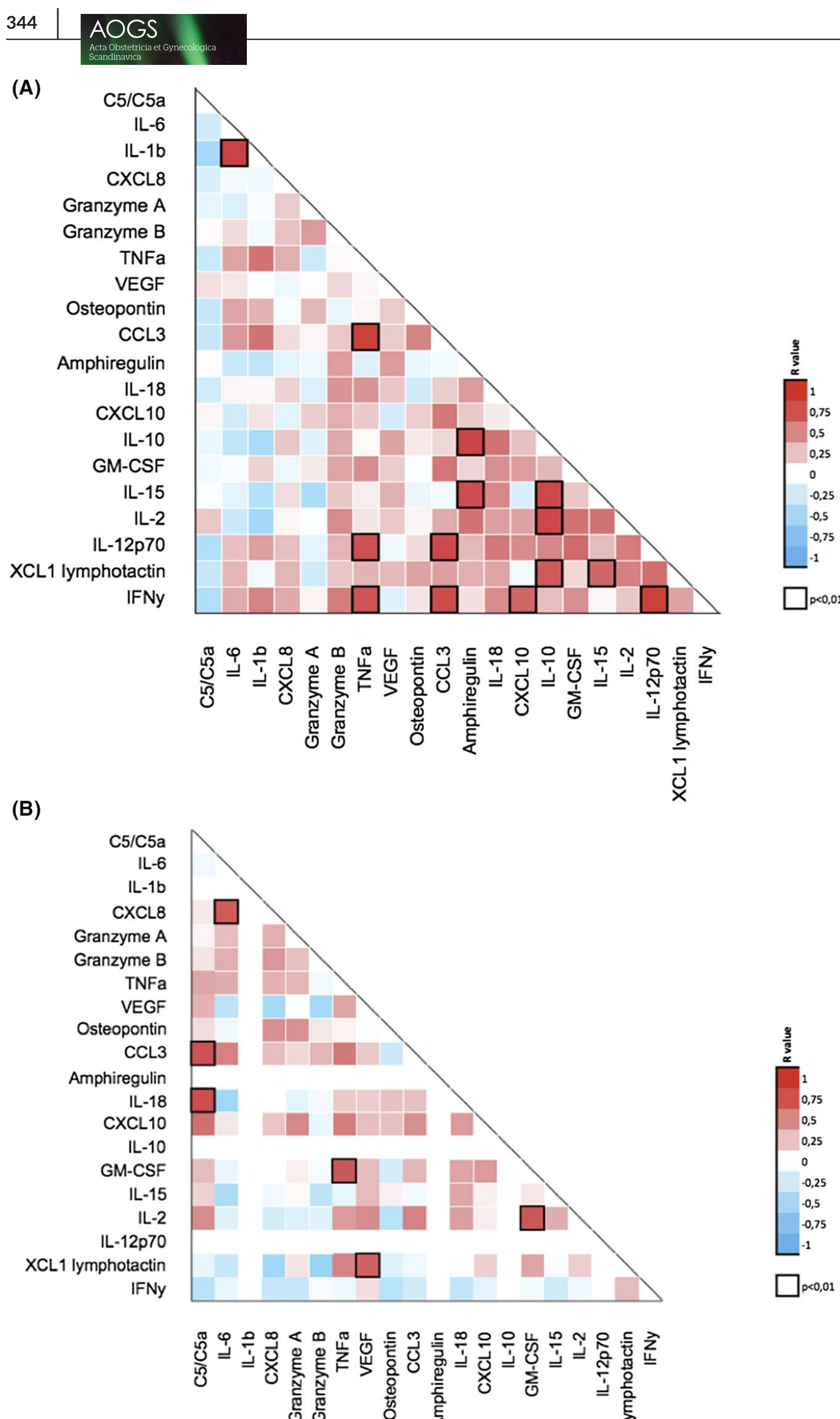

FIGURE 3 Correlations of cytokines in menstrual blood plasma (A). Correlations of cytokines in peripheral blood plasma (B). Correlations framed in black are statistically significant $P < .01$. C5/C5a, complement component 5; CCL3, chemokine (C-C motif) ligand 3; CXCL, chemokine (C-X-C motif) ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; XCL1, chemokine (C motif) ligand 1 [Color figure can be viewed at wileyonlinelibrary.com]

Granzyme A and B were also detected in high concentrations. These enzymes are potent pro-apoptotic factors that initiate caspase-driven cell death pathways, present in cytotoxic T and NK cell granulae.[31,32] Yet, in menstrual blood they were detected to the same extent as in the lyzed external control, which might attribute the levels of these factors to cell lysis (Figure 1A,E). Interestingly, IL-15, known to promote uterine NK cell proliferation,[33] was found in a relatively low concentration, but significantly higher than in peripheral blood. This was also true for the known NK-cell-activating cytokines IL-12p70 and IFN-γ. As menstruation is marked by ongoing inflammation of the uterine cavity,[1] it is expected that C5/C5a, IL-6, IL-1β, CXCL8, and granzyme A and B are present in high concentrations in menstrual blood. In addition, NK cells are the most abundant among leukocytes of the uterus during the mid-luteal phase, but then decrease in frequency towards the menstrual phase.[34,35] Therefore, the low concentrations found in this study of IL-15, IL-12p70, and IFN-γ, as well as XCL1/Lymphotactin, seem to follow those patterns of immune cell fluctuations throughout the cycle.

In contrast to the similarity between participants regarding the menstrual blood plasma cytokine profile, we could detect a distinct

difference in the cytokine profile between menstrual and peripheral blood plasma. The greatest differences were found in the pro-inflammatory cytokines IL-6, IL-1β, and CXCL8, but the most striking observation was the distinct pattern regarding concentrations and correlations in the cytokine profiles in blood plasma collected from the different compartments. In both menstrual and peripheral blood plasma, we could observe distinct patterns in correlations, indicating a different method of activation, and underlining the strong difference between these compartments.

To test whether iatrogenic wounding of the endometrium in the mid-luteal phase would evoke a change in the menstrual blood plasma cytokine profile we performed an endometrial scratching 7-9 days after the luteinizing hormone surge. To our knowledge, no previous studies have been designed to study the menstrual blood cytokine profile after endometrial scratching at this time-point of the menstrual cycle. Comparisons with other studies are thereby limited. It has previously been reported that endometrial scratching in mid to late follicular phase causes elevated levels of pro-inflammatory cytokines such as TNF, IL-15, growth-related oncogene-α, and macrophage inflammatory protein 1β,[36] and some studies have suggested that it may also improve live-birth rate if performed in the same cycle of women undergoing intrauterine inseminations.[37] However, a recent large randomized multicenter trial for endometrial scratching before in vitro fertilization could not detect a benefit for those women undergoing endometrial scratching, at least not in terms of live-birth rate.[17] In our study, the cytokines were not analyzed directly after endometrial scratching, but in the following menstrual phase. Here, we could not detect any difference in cytokine profiles, including levels of pro-inflammatory cytokines. We noted that whatever change in the uterine cytokine expression an endometrial scratch evokes, it is resolved already before the next menstrual shedding. This is a reassuring finding and fits with the common belief that the endometrium heals rapidly after injury.[38] The timing for the endometrial scratching in mid-luteal phase may constitute a problem, making our study result difficult to compare to other studies that have performed the endometrial scratching in follicular phase. We have also included only healthy nulligravidae in our study and the results shown here may not be comparable with women suffering from infertility. However, our study is the only study to investigate the cytokine profile in menstrual blood after endometrial scratching.

The relatively small sample size may be a limitation to this study. On the other hand, the careful selection of healthy controls and the thorough clinical collection of data with pre-set time-points for intervention (7-9 days after luteinizing hormone rise) and sampling constitutes a strength. Furthermore, the laboratory experimental work was carried out on the same plate to reduce intra- and inter-experimental bias between batches. High levels of granzymes A and B in both menstrual blood plasma and external control peripheral blood plasma (Figure 1E sample 3), may be the result of leakage of cytotoxic granules as a direct consequence of cell lysis. Osteopontin on the other hand, is known to degrade into smaller protein fragments upon freezing and thawing.[39] Based on this, we can conclude that the time between sampling and analysis, as well as treatment of the sample, can affect the results and should be taken into account in future studies. In this case, this might give rise to a potentially false high concentration of granzymes and a false low concentration of osteopontin in menstrual blood plasma. Nevertheless, the majority of cytokines can be measured reliably in menstrual blood.

5 | CONCLUSION

We have described a distinct cytokine pattern of menstrual blood plasma completely different to peripheral blood. This is one important step towards understanding the mechanisms underlying the endometrial shedding and regeneration. Our data suggest that any change in cytokine expression, presumably evoked by mid-luteal phase endometrial scratching, resolves before menstruation and is not detected when comparing menstrual blood plasma cytokines before and after such intervention.

The characterization of menstrual blood cytokine profiles in healthy women found in this study can be used in further comparisons with pathological states where an altered cytokine profile may be involved, such as endometriosis and repeated implantation failure.

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CONFLICT OF INTEREST

None.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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