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Diminished Frequency of Menstrual and Peripheral Blood NKT-Like Cells in Patients With Unexplained Recurrent Spontaneous Abortion and Infertile Women

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Abstract

Systemic monitoring of immune system may not precisely outline the local immune status in the uterus. This survey is a continuation of our previous studies on potential usefulness of menstrual blood (MB) immunophenotyping as a tool for investigation of immunological disturbances in pregnancy-related disorders. Peripheral blood (PB) and MB from healthy fertile (n = 15), unexplained recurrent spontaneous abortion (URSA; n = 15), and unexplained infertile women (n = 8) were collected simultaneously in the second day of their menstrual cycle and frequency of natural killer T (NKT)-like cell subpopulations were assessed by flow cytometry. Menstrual blood of all experimental groups contained higher percentage of TCR$\alpha\beta^+$, CD45RO$^+$, and CD16$^-$ NKT-like cells compared to corresponding PB. Frequency of MB NKT-like cells in unexplained infertile participants was lower than fertile and URSA groups. Compared to normal participants, patients with URSA had lower frequency of PB TCR$\alpha\beta^+$ and higher CD16$^+$, while in infertile woman frequencies of PB CD45RO$^+$, CD45RO$^-$, CD16$^-$, IL17$^+$, and MB CD45RO$^+$ NKT-like cells were lower. Although, PB and MB seemingly have the same histological nature, our results showed that MB contained different composition of NKT-like subsets with different cytokine profiles and could be viewed as one potential biological sample for evaluation of patients with infertility and URSA.

Keywords

abortion, immunophenotype, infertility, menstrual blood, NKT-like cells

Introduction

The uterus has unique microenvironment compatible for establishment of pregnancy and development of semiallogeneic fetus.[1] In this context, alterations in this microenvironment pertaining thrombophilic, endocrine, anatomic, infectious, and immune-related factors could potentially lead to pregnancy failure.[2] Although in about 50% of women with recurrent spontaneous abortion (RSA) and 25% to 30% of all infertility cases, no clear etiology could be found,[3,4] local or systemic imbalance of the immune system has been reported to be a causative factor in a great portion of pregnancy disorders mentioned above.[5-7] There are plenty of reports showing profound impact of such immune cells as NK and T-cells on the course of pregnancy,[8,9] but potential role of natural killer T (NKT) cells in this process has not still been well-defined. Natural killer T-cells have a substantial impact on immune hemostasis by regulating adaptive and innate arms of immune system.[10,11]

Natural killer T-like cells are a small subset of T-cells that were originally defined in humans based on coexpression of NK cell marker, CD56, and pan T-cell molecule, CD3.[12]

In this study, we analyzed CD3$^+$CD56$^+$ NKT-like cells. Notably, this population may also contain mucosal-associated invariant T cells (MAIT) cells,[13] γ-δ-T cells,[14] conventional CD8$^+$ T cells,[15] and NKT cells.[16] Nonetheless, it was not within the scope of the current study to separately analyze each

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individual T-cell subsets mentioned above, so we collectively defined $CD45^+CD3^+CD56^+$ as NKT-like cells.

These are a specialized population of T-cells that express a semi-invariant T-cell receptor (TCRαβ). The specialized feature of TCR on NKT cells is that it recognizes glycolipid antigens presented by the MHC I-like molecule CD1d. Most NKT cells, known as type I NKT cells, express an invariant TCR α chain and one of a small number of TCR β chains and the majority of these cells express TCRαβ.[16] This cell population has been reported to have a role in infection, cancer, and transplantation through production of cytokines.[17,18] Reportedly, effector function of NKT cells especially in patients with cancer is in part affected by the expression of CD16.[19]

Indeed, $CD16^+$ NKT-like cells are a major source of many pro-inflammatory mediators.[20,21]

Upon T-cell receptor (TCR) activation, NKT-like cells have the ability to rapidly produce large amounts of both pro- and anti-inflammatory mediators, such as Interferon-gamma (IFNγ), interleukin 4 (IL4), and interleukin 17 (IL17) without need for priming or clonal expansion and thus influence diverse immune responses and pathogenic processes.[20,22] Accordingly, NKT-like cells can paradoxically suppress or stimulate immune responses depending on microenvironment they reside.[23] It has been demonstrated that adult human NKT-like cells express enhanced levels of the memory marker, CD45RO, compared with α/β T-cells.[24]

It was reported that the percentages of peripheral blood (PB) NKT cells are significantly increased in pregnancy compared to nonpregnancy state.[25] Indeed, higher frequency of NKT cells has been found in decidua of pregnant women compared to their PB.[26] Therefore, it is conceivable to imagine that NKT-like cells play an important role in implantation success and fetal tolerance through modulation of innate and adaptive immunity.[27] In support of this assumption, it has been reported that abnormal function and frequency of NKT cell subsets in PB and decidua are associated with RSA and implantation failure after In vitro fertilization (IVF).[28,29]

Despite being the gold standard specimen for investigation of local immune microenvironment of the uterus, endometrium has its own limitation by profound changes in immune cell density and subtypes during follicular and secretory phases under the influence of ovarian steroid hormones. Indeed, endometrium sampling is per se an invasive technique.[30] We recently showed that menstrual blood (MB) could be viewed as one biologically relevant sample to be studied in patients with recurrent abortion or infertility.[31,32] It is an accessible sample without the need for invasive techniques for sampling. Indeed, refreshing nature of MB makes multiple sampling possible. With this in mind, we investigated frequency of PB and MB NKT cell subsets in women with unexplained RSA (URSA) and unexplained infertility in reference to fertile individuals.

Materials and Methods

Study Population

In this study, we enrolled 15 healthy fertile women with at least one successful term pregnancy without medical intervention as a control group, 15 women with URSA (defined as at least 2 successive unexplained miscarriages before 20 weeks of gestation) and 8 women with unexplained infertility (defined as the inability to conceive after 12 months of unprotected sexual intercourse) with regular menstrual cycle. In patients with URSA, specimens were collected at least 3 months after abortion. Mean age, mean duration of menstrual cycle, body mass index, and number of children or abortions in the 3 groups are summarized in Table 1.

Table 1. Summary of the Mean Age, Mean Duration of Menstrual Cycle, Body Mass Index (BMI), and Mean Number of Children/Abortion in Fertile, Unexplained Recurrent Spontaneous Abortion (URSA), and Infertile Women.

Variable	Fertile (n = 15)	URSA (n = 15)	Infertile (n = 8)	*P*
Age, years	35.4 (4.4)	31.02 (4.6)	35.00 (6.96)	NS
Menstrual cycle duration, day	28.6 (2.3)	27.8 (2.5)	27.87 (1.45)	NS
BMI, kg/m^2	24.9 (2.5)	26.7 (2.8)	25.79 (4.02)	NS
Number of children	1.3 (0.4)	–	–	
Number of abortion	–	2.4 (0.6)	–	

Abbreviations: NS, nonsignificant; URSA, unexplained recurrent spontaneous abortion.
[a]Data are expressed as mean (SD).

Normal control women were chosen from health workers in Avicenna Research Institute (ARI) and Avicenna Infertility and Recurrent Abortion Clinic (AIC). All control samples were collected and assessed in parallel to patient samples. Indeed, the participants in control and experimental groups studied in this research project were the same participants we studied elsewhere.[31-33]

Unexplained RSA and infertile women were chosen after thorough clinical and laboratory investigations from couples attending to AIC. All patients with etiologies known for RSA or infertility were excluded including uterine abnormality, endocrinologic dysfunction, abnormal karyotype, positive results for autoantibodies associated with infertility or abortion, and presence of thrombophilic state as judged by thrombophilia laboratory tests and male factor.[32] The study was approved by institutional review board and the ethics committee for medical research of ARI. The study purpose and procedures were carefully explained, and an informed consent was obtained from all women willing to participate.

Blood Collection

Collection of MB was performed as reported earlier.[33] In brief, 10 mL heparinized PB and 5 to 15 mL MB was collected in the second day of menstrual bleeding. Menstrual blood was transferred into a sterile 50 mL falcon tube containing heparin, fungizone, and penicillin/streptomycin and transported to the laboratory in cold chain.

Table 2. Antibodies Used in This Study.

mAb	Fluorochrome	Clone
CD45	PE	HI30
CD3	PE-CY5	UCHT1
CD56	FITC	MEM188
CD16	PE	CB16
TCRαβ	PE	IP26
CD45RO	PE	UCHL1
IFNγ	PE	4S.B3
IL4	PE	8D4-8
IL17A	PE	eBio64DEC17
Isotype IgG1κ	FITC	P3.6.2.1
Isotype IgG1	PE	P3.6.2.1
Isotype IgG1	PE-CY5	P3.6.2.1
Isotype $IgG2_a$ κ	FITC	eBM2a

Abbreviations: mAb, Monoclonal antibodies; IFNγ, Interferon-gamma; IL4, interleukin 4; IL17, interleukin 17, FITC, Fluorescein Isothiocyanate; PE, Phycoerythrin.

Mononuclear Cell Isolation

Menstrual blood from all groups were washed twice with cold phosphate buffered saline (PBS) and passed through a 70-μM nylon cell strainer (Becton Dickinson Falcon, Franklin Lakes, New Jersey) to remove large clots and strings of mucus. Peripheral blood mononuclear cells and MB mononuclear cells (MBMC) were separated using Histopaque (Sigma-Aldrich, St. Louis, Missouri) density-gradient centrifugation as described elsewhere.[32] Isolated MNCs were washed twice with RPMI-1640 and checked for viability to be more than 95% by trypan blue exclusion test. We also performed apoptosis detection test as judged by PI and Annexin V staining for PB and MB cells from all patients and healthy individuals before stimulation using the method reported earlier.[33] In almost all cases, viability of the collected cells were more than 95%, and no significant presence of apoptosis was detected in any of the individuals.

Flow Cytometry

For flow cytometry analyses of MB and PB of each individual, the expression of 6 markers (TCRαβ, CD45RO, CD16, IFNγ, IL4, and IL17) in $CD3^+CD56^+$ NKT-like cells was separately assessed. In this regard, triple staining of anti-CD3-PE-CY5, anti-CD56-FITC, and PE-conjugated antibody against one of the markers specified above were performed separately in 6 individual tubes. The antibodies used in this study (all from eBioscience, San Diego, California) are listed in Table 2. Briefly, MNCs were washed twice with wash buffer (PBS containing 1% fetal bovine serum), resuspended in the same buffer (1×10^6 cells/100 μL), and stained with fluorochrome-conjugated antibodies directed against CD45, CD3, CD56, CD16, CD45RO, and TCRαβ for 45 minutes in the dark at 4°C.

For detection of intracellular IFNγ, IL4, and IL17 cytokines, mononuclear cells (MNC) were first stimulated for 6 hours in the presence of phorbol 12-myristate 13-acetate (25 ng/mL; Sigma) plus ionomysin (500 ng/mL; Sigma) and monensin (1 μM; BD BiosciencesSan Jose, CA, USA). Cells were then washed twice with washing buffer and stained with anti-CD56 and CD3 antibodies for 45 minutes at 4°C in the dark. In the next step, cells were washed, fixed, and permeabilized with fixation/permeabilization buffer as recommended by the supplier (BD Biosciences). Cells were then incubated with optimized concentration of fluorochrome-conjugated antibodies against IFNγ, IL4, or IL17 mAb for 45 minutes at 4°C in the dark.

Due to the presence of considerable numbers of red blood cells (RBC) in MBMC fraction, RBC lysing step was performed before flow cytometric analysis (DAKO, Denmark, S3325). Analysis was done by a Partec flow cytometer (Nuremberg, Germany), and FloMax software (Partec, Münster, Germany) was used for data analysis. To analyze subsets with a very small percentage, approximately 100 000 cells were analyzed for each sample. Due to the presence of variety of cells including endometrial epithelial and stromal cells in isolated MBMC fraction, lymphocyte gating could not be easily and precisely performed solely based on side-forward scatter plot. To this end, for each sample, the gate encompassing more than 90% of lymphocytes was first determined using anti-CD45 mAb, and all subsequent analyses were performed on this gate. Immune cell subsets were quantified based on the percentage of triple markers. In PB and MB of all experimental groups, the frequency of NKT-like ($CD3^+CD56^+$) cell subsets were assessed in CD3 gated cells for the expression of TCRαβ, CD45RO, CD16, IFNγ, IL4, and IL17. Representative flow cytometry dot plots illustrating the analysis method for detection and enumeration of subsets are shown in Figure 1.

In brief, gating strategy was as follows: (1) gating of CD45 cells (R1), (2) gating of $CD3^+$ cells in CD45 gate (R2), and (3) In the child flow chart, coexpression of CD56 and the marker of interest was then evaluated.

Statistical Analyses

Fifteen women from each fertile and URSA groups and 8 unexplained infertile women were enrolled to this study. Due to technical problems, the number of samples examined in some experiments was varied. In paired comparisons (ie, MB vs PB of the same group), results were compared only when data from both specimens were available. Statistical analysis was performed using Statistical Package for the Social Sciences for windows (SPSS Inc, Chicago, Illinois). Graphs were plotted by Graphpad Prism software 5.0 (San Diego, California). Categorical data were expressed as number and percentage, and numerical data as mean (SD). Wilcoxon rank test was performed for comparing data from the paired samples (PB and MB) of the same individuals. Statistical differences between 2 groups were determined by nonparametric Mann-Whitney U test. P values less than .05 were considered statistically significant.

Results

Comparison of NKT-Like Cells (Intergroup, Intragroup Analyses)

Frequency of NKT-Like Cells. Natural killer T-cells have a substantial role on immune regulation through modulation of

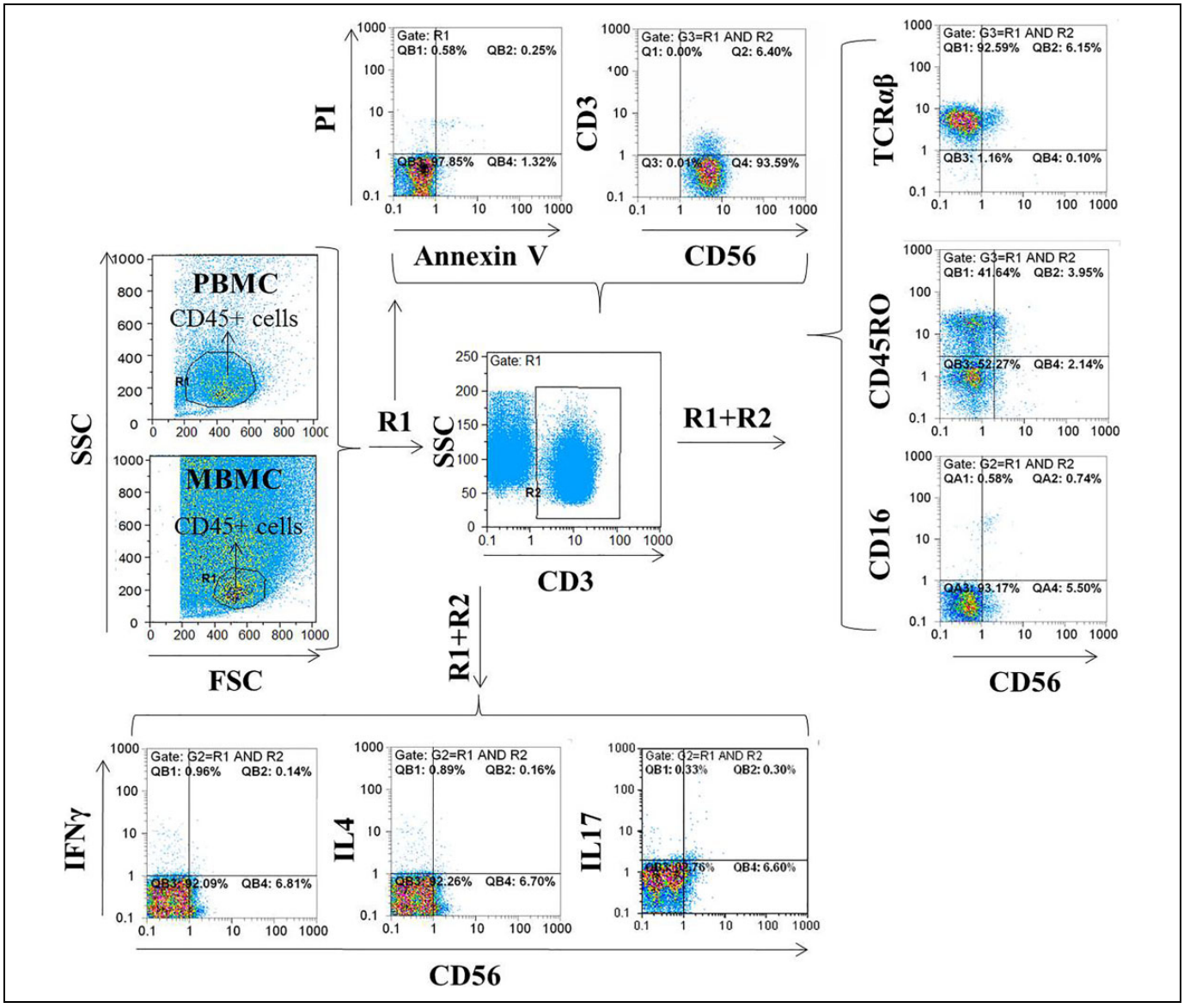

Figure 1. Representative flow chart of peripheral blood (PB) and menstrual blood (MB) immunophenotyping demonstrating sequential gating method. Initial gating (R1) was performed on forward versus side scatter (FSC/SSC) to determine CD4-positive cells (R1) as described in Materials and Methods. $CD3^+$ (R2) was then gated on R1 and separately used for enumeration of cells. CD indicates cluster of differentiation; IFN, interferon; IL, interleukin; MBMC, menstrual blood mononuclear cells; PBMC, peripheral blood mononuclear cells; TCR, T-cell receptor.

innate and adaptive arms of immune system, and their role in the course of pregnancy has been the focus of recent studies. In this regard, we first analyzed the differential frequency of these cells in 3 groups of study. The frequency of $CD3^+CD56^+$ NKT-like cells in PB and MB of all experimental groups was measured and analyzed. The results showed that the percentage of NKT-like cells in MB of normal controls and URSA women ($P = .03$, .003, respectively) were significantly higher than those in corresponding PB (Figure 2B). Also, percentage of MB NKT-like cells in unexplained infertile participants was lower than fertile and URSA groups ($P = .001$, .003, respectively; Figure 2C).

Expression of TCRαβ. The majority of NKT-like cells express TCRαβ on their surface. We next examined whether or not expression of this marker is affected in women with infertility or RSA. Our results showed that MB of all normal participants, URSA, and infertile women contained significantly higher percentage of $TCR\alpha\beta^+$ NKT-like ($CD56^+CD3^+$) cells as compared to corresponding PB ($P = .01$, .003, and .04, respectively; Figure 3B). Compared to normal participants, PB of patients with URSA had lower frequency of the $TCR\alpha\beta^+$ NKT-like cells ($P = .04$; Figure 3C).

Expression of CD45RO. Differential expression of CD45RO as the effector/memory marker was then examined in all groups.

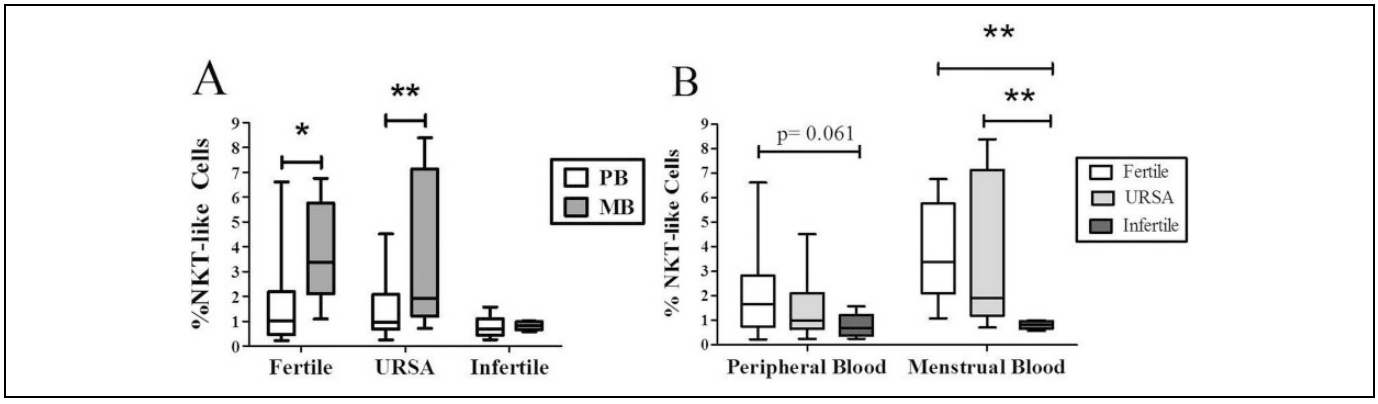

Figure 2. Comparison of $CD3^+CD56^+$ natural killer T (NKT)-like cells in peripheral blood (PB) and menstrual blood (MB) of fertile, URSA, and unexplained infertile women. A, Intragroup comparison (n = 10, 12, and 6 from left to right) and (B) Intergroup comparison (n = 15, 15, 8 and 10, 12, 6 from left to right). Frequency of cells was calculated in CD3 gate. Results are shown as box-and-whisker plots (5-95 percentiles). Horizontal lines represent medians. URSA indicates unexplained recurrent spontaneous abortion, $^*P \leq .05, .01$; $^{**}P \leq .01$.

In overall, the percentage of $CD45RO^+$ and $CD45RO^-$ NKT-like cells was higher in MB compared to PB of all groups. These differences reached to a statistically significant level in normal group for $CD45RO^+$ ($P = .03$) and in URSA patients for $CD45RO^+$ and $CD45RO^-$ NKT-like cells ($P = .01$ and .002, respectively; Figure 4B). In addition, intergroup analysis revealed a higher frequency of PB $CD45RO^+$ and $CD45RO^-$ NKT-like cells of normal controls compared to unexplained infertile patients ($P = .01, .05$). The same trend was also observed for MB $CD45RO^+$ NKT-like cells ($P = .05$; Figure 4B and C).

Expression of CD16 Marker. According to some reports, function of NKT-like cells is affected by the expression of CD16. Therefore, we were interested in learning whether infertile and RSA patients have differential expression of this marker on their NKT-like cells.

Although no statistical difference was observed in frequency of MB compared to PB $CD16^+$ NKT-like cells, $CD16^-$ NKT-like cells were significantly higher in MB of all groups compared to the their corresponding PB ($P = .05, .002$, and .01, respectively; Figure 5B). Also the ratio of $CD16^-/CD16^+$ NKT-like cells was found to be significantly higher in MB compared to PB of fertile and URSA patients ($P = .01$ and .03, respectively; Figure 5C). Interestingly, URSA participants contained higher frequency of PB $CD16^+$ NKT-like cells compared to control group ($P = .02$), while unexplained infertile participants showed lower frequency of PB $CD16^-$ NKT-like cells as compared to control women ($P = .04$; Figure 5D). Moreover, the ratio of $CD16^-/16^+$ NKT-like cells were lower in PB of URSA and infertile patients ($P = .003$ and .02, respectively) as well as in MB of URSA patients compared to control women ($P = .05$; Figure 5E).

Expression of Intracellular IFNγ, IL4, and IL17. Cytokines are major modulators of immune system at the feto-maternal interface. Therefore, we tested the expression of cytokines associated with T1, T2, and T17 phenotype in NKT-like cells. The percentage of $IFN\gamma^+$ NKT-like cells in MB of fertile and URSA ($P = .02$ and .01, respectively) were significantly higher than those in corresponding PB. Furthermore, higher frequency of $IL17^+$ NKT-like cells was found in MB of fertile and unexplained infertile women as compared to PB ($P = .02$ and .04, respectively), while no statistical difference was observed in aforesaid subset in MB and BP of patients with URSA. Akin to $IFN\gamma^+$ NKT-like cells, frequency of $IL4^+$ NKT-like cells were also found to be higher in MB of URSA patients compared to PB of the same patients ($P = .02$; Figure 6B). Also PB $IL17^+$ NKT-like cells in unexplained infertile participants exhibited lower frequency than normal control and URSA group ($P = .01, .007$, respectively; Figure 6C).

Discussion

Considering the key contribution of immune system in such pregnancy disorders as recurrent abortion and infertility, there has been growing efforts to find immunological biomarkers for diagnosis of immune-mediated infertility and abortion.[34] In this regard, PB and endometrium comprise the most common specimens investigated so far. Recently, we have shown the utility of MB for monitoring of local immune system in infertile and URSA patients.[31,32] Here, we studied by flow cytometry the differential frequency of $CD3^+CD56^+$ NKT-like cells in MB and PB of infertile and URSA patients in reference to normal fertile controls. There is limited and conflicting data on potential contribution of NKT-like cells in infertility and abortion.

Yuan et al showed that percentage of PB and decidual NKT-like cells were significantly higher in URSA patients than in normal pregnant and nonpregnant women, although their results are somehow out of our expectation, because they reported that majority of decidual T-cells express CD16 and that about 60% of decidual $CD3^+$ T-cells express either CD56 or CD16.[27] Conversely, Yamamoto et al found lower percentages of decidual NKT-like cells in patients with RSA.[29] We observed similar frequency of NKT-like cells in MB of normal

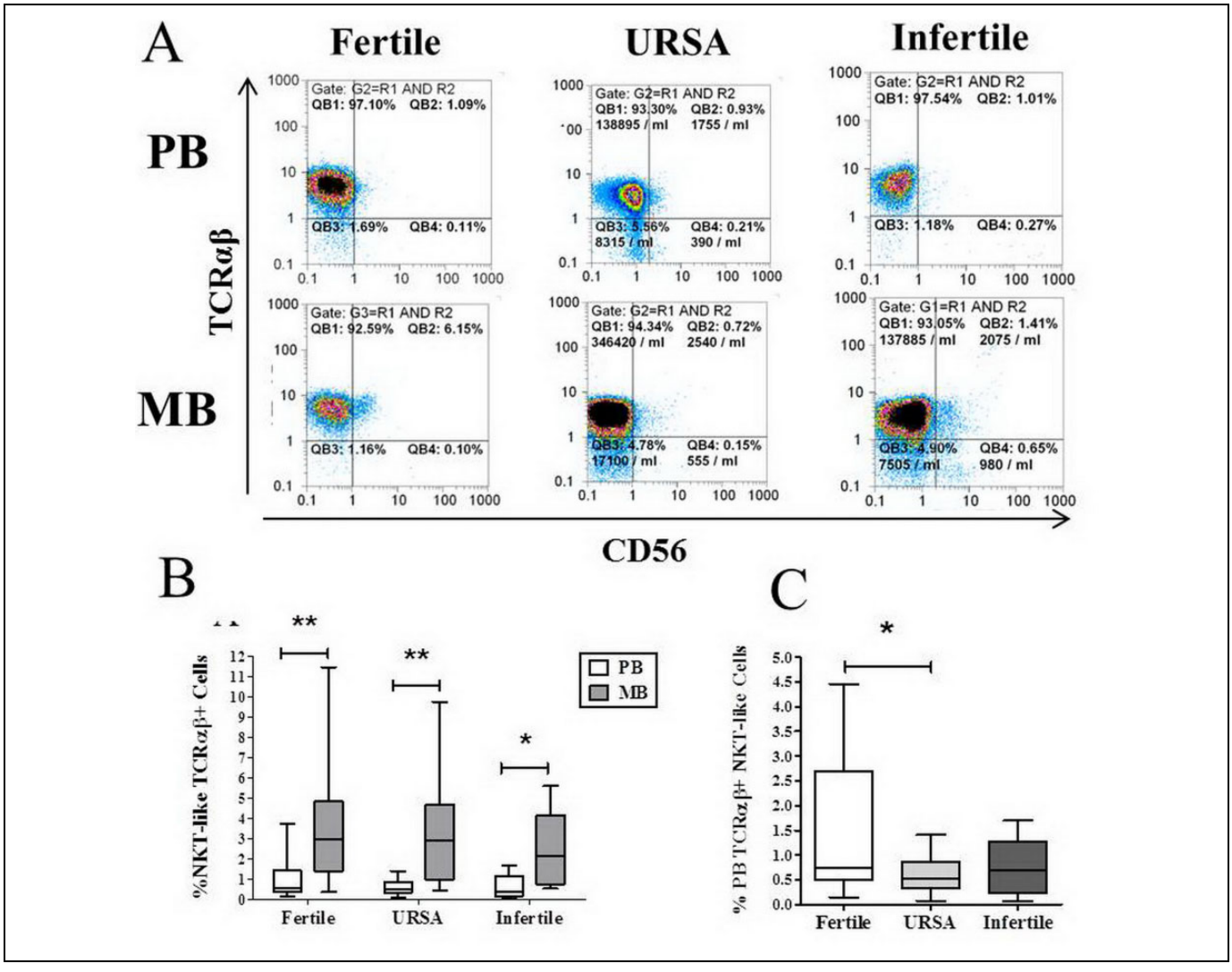

Figure 3. Comparison of TCRαβ natural killer T (NKT)-like cells in peripheral blood (PB) and menstrual blood (MB) of fertile, URSA, and unexplained infertile women. A, Representative flow cytometry plots for marker analysis in the 3 groups of study. B, Intragroup comparison (n = 11, 11 and 6 from left to right). C, Intergroup comparison of peripheral blood TCRαβ$^+$ NKT-like cells frequency (n = 15, 15 and 8 from left to right). Frequency of cells was calculated in CD3 gate. Results are shown as box-and-whisker plots (5-95 percentiles). Horizontal lines represent medians. URSA indicates unexplained recurrent spontaneous abortion, *$P \leq .05$, 0.01; **$P \leq .01$.

fertile and URSA women, which were significantly higher than those in corresponding PB. Of note, infertile women had comparatively lower percentage of both PB and MB NKT-like cells pointing to the prominent role of this cell population in initiation of pregnancy. NKT cells were found to have a critical role in the development of systemic tolerance associated with immune privileged sites and induction of self-tolerance through increase in regulatory T-cells population.[35]

Higher frequency of MB versus PB NKT-like cells in healthy women was also reported earlier by van der Molen et al although they failed to find a significant difference.[36] Lower frequency of MB NKT-like cells in infertile women may be due to lower frequency of this cell type in PB that could negatively affect recruitment of these cells to the endometrium. Such explanation has also been given for reduced numbers of decidual CD56$^+$ cells in infertile women.[37]

At the PB level, Yahata et al reported lower frequency of CD56$^+$CD3$^+$ NKT-like cells in PB of nonpregnant patients with a history of RSA compared to those obtained from normal nonpregnant women.[38] Nonetheless, no statistical difference of PB NKT-like cells in patient with RSA and control group was reported by Lee et al.[39] Such inconsistent results may in part be explained by different criteria used to define RSA with unknown etiology, different number of study population, different entity of samples (MB vs decidua), and more importantly, timing of sampling in menstrual cycle which could profoundly affect the frequency of immune cells.

Also we showed that majority of PB or MB NKT-like cell express TCRαβ irrespective of study group and that statistical differences in intergroup or intragroup analyses of CD3$^+$CD56$^+$ NKT-like cells are also the case for CD3$^+$CD56$^+$ TCRαβ$^+$ cells.

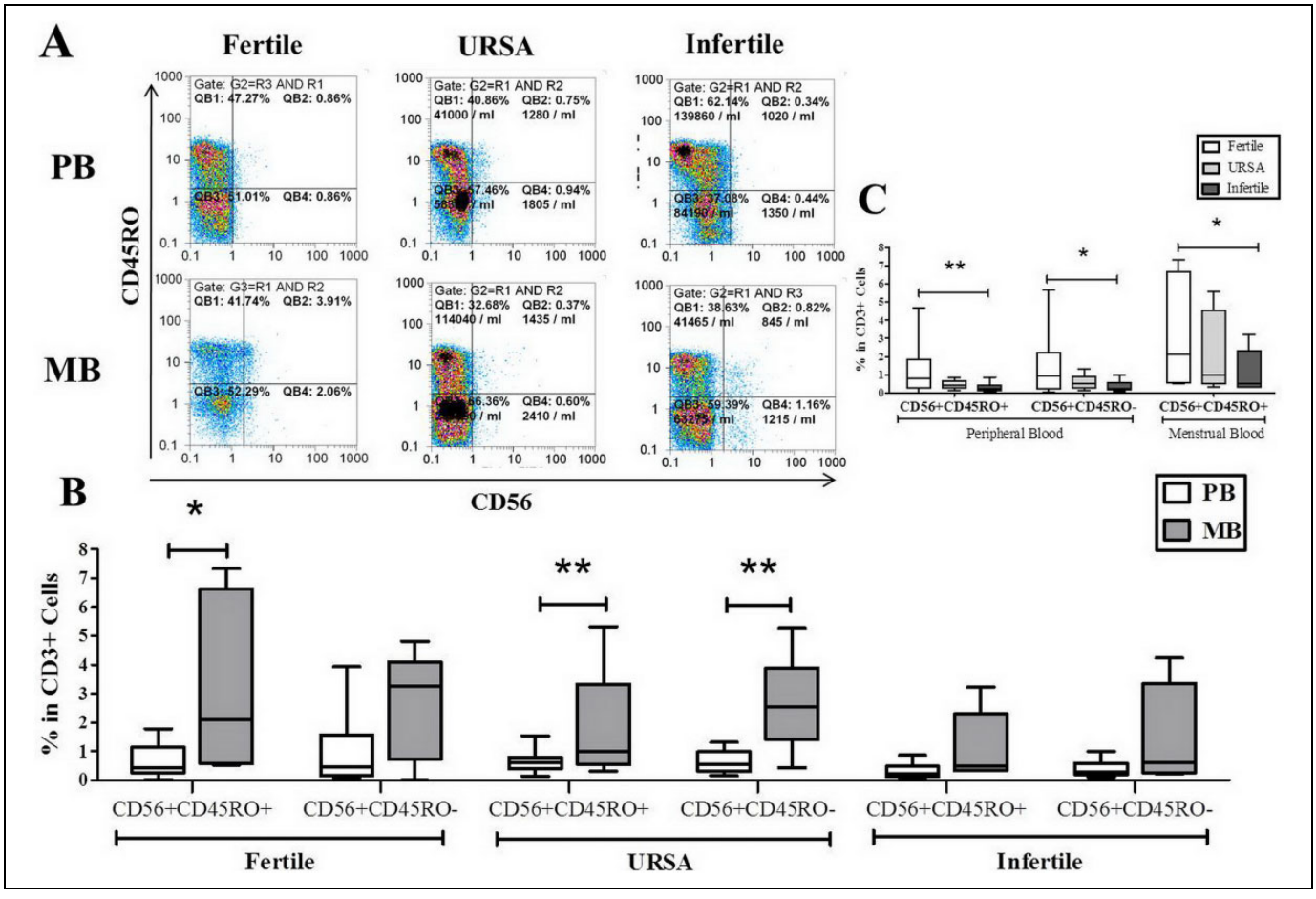

Figure 4. Comparison of peripheral blood (PB) and menstrual blood (MB) frequency of $CD45RO^+$ and $CD45RO^-$ natural killer T (NKT)-like cells in fertile, URSA, and unexplained infertile women. A, Representative flow cytometry plots for marker analysis in 3 groups of study. B, Intragroup comparison (n = 10, 10-10, 12 and 7, 7 from left to right), and (C) Intergroup comparison (n = 14, 13, 8-13, 15, 8 and 10, 12, 8 from left to right). Frequency of cells was calculated in CD3 gate. Results are shown as box-and-whisker plots. Horizontal lines represent medians. URSA indicates unexplained recurrent spontaneous abortion, $*P \leq .05$; $**P \leq .01$.

There is paucity of data on fluctuation of PB and endometrial NKT-like cells during menstrual cycle. Flynn et al reported that the frequency of endometrial $CD3^+CD56^+$ cells were increased at the late secretory compared to late proliferation phase.[40] With commencement of pregnancy, the percentage of this cell type is increased in early pregnancy decidua compared with PB pointing to the eminent role of NKT-like cells in early pregnancy period.[16,41-43] Our findings on increased levels of NKT-like cells in MB compared to PB in all groups of study further support the previous reports denoting enrichment of this cell type in late secretory endometrium.

We observed that the frequency of MB $CD45RO^+$ NKT-like cells was higher compared to their PB counterpart in fertile and URSA women, and the same trend was also observed in women with unexplained infertility. Also in URSA group, $CD45RO^-$ NKT-like cells significantly increased in MB than PB. High frequency of NKT-like cells expressing this primed/memory marker in MB could be linked to their vital role in the regulation of immune responses and in maintenance of self-tolerance in the uterine through the induction of regulatory T-cells.[35] In addition, de Lalla et al showed that invariant NKT (iNKT) cells homogeneously express the primed-memory marker, CD45RO.[44] We showed that only about half of the MB or PB NKT-like cells in all groups of study express CD45RO. The reason behind this inconsistency may be due to mixed NKT cell subtypes (NKT-like cells and iNKT cells) we studied here. Human iNKT cells are a subset of T-cells that express both NK cell receptor (CD56) and a rearranged TCR which has a semi-invariant TCRα chain (Vα24-Jα18). Upon activation, iNKT cells rapidly secrete regulatory cytokines that can modulate the activity of other immune cells such as NK cells, dendritic cells, macrophages, neutrophils, B cells, and T-cells.[45] In this context, they are often considered as a "bridge" between the innate and adaptive immune systems. Decreased levels of $CD45RO^+$ NKT-like cells in MB and PB of infertile women could be viewed as one potential mechanism leading to implantation failure, although the mechanism of such potential association is not clear for us.

We also observed that in MB of all groups, $CD16^-$ NKT-like cells are more frequent compared to those NKT-like cells

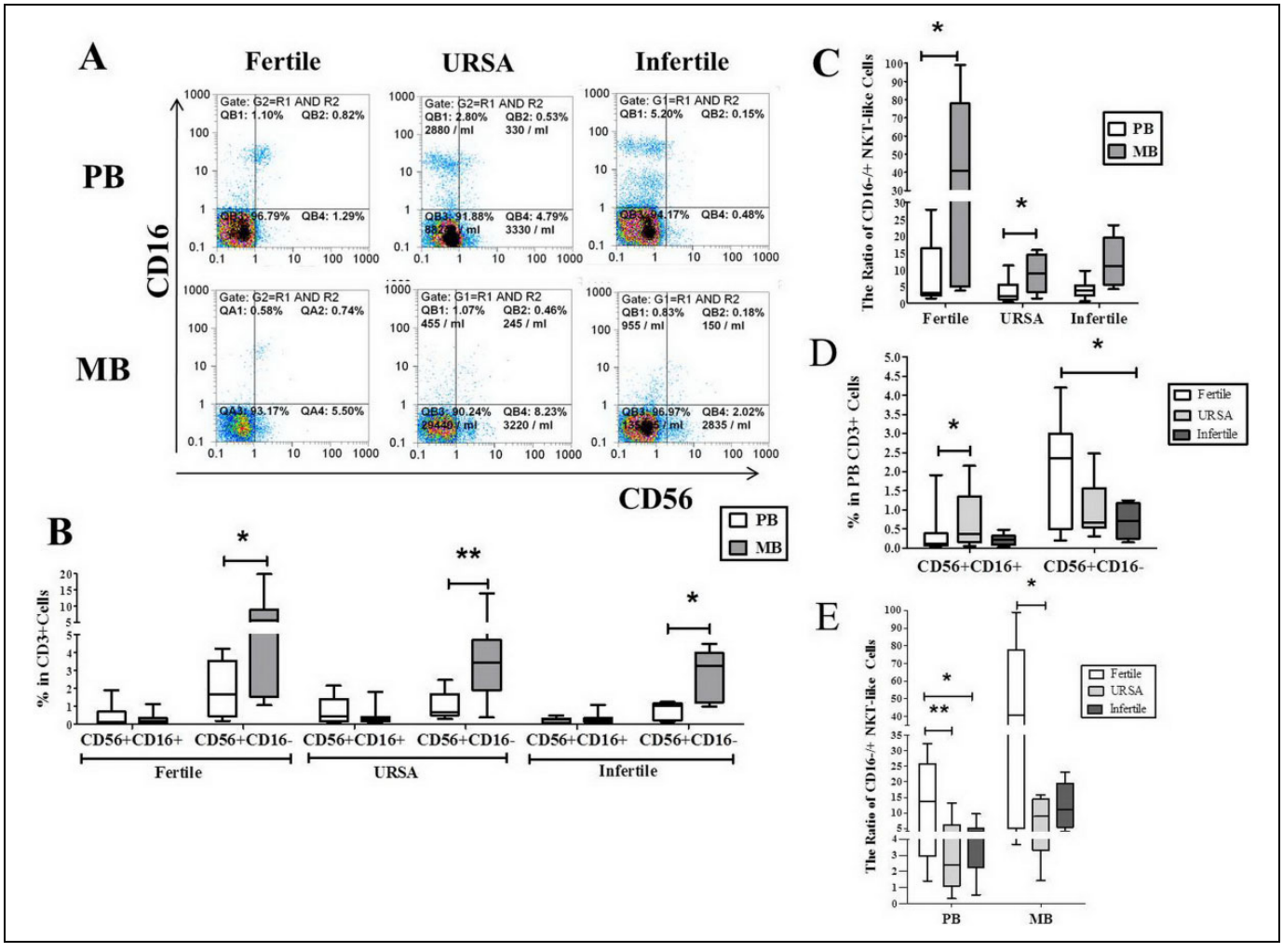

Figure 5. Comparison of peripheral blood (PB) and menstrual blood (MB) frequency of $CD16^+$ and $CD16^-$ natural killer T (NKT)-like cells in fertile, URSA, and unexplained infertile women. A, Representative flow cytometry plots for marker analysis in 3 groups of study. B, Intragroup comparison (n = 9, 14, and 7 from left to right), (C) Intragroup comparison of $CD16^-/CD16^+$ ratio (n = 7, 12 and 7 from left to right), (D) Intergroup comparison of PB (n = 15, 15, 8-15 and 15, 8 from left to right), and (E) Intergroup comparison of PB and MB $CD16^-/CD16^+$ ratio (n = 14, 15, 8-7, 12, 7 from left to right). Results are shown as box-and-whisker plots (5-95 percentiles). URSA indicates unexplained recurrent spontaneous abortion, $^*P \leq .05$; $^{**}P \leq .01$.

expressing CD16. Our finding on higher frequency of $CD16^-$ NKT-like cells in MB is similar to what have been reported for uterine NK cells.[46] Based on several reports, NKT-like cells expressing CD16 have effector function and their frequency positively correlates with prognosis and tumor-free survival in patients with B-cell lymphoma and chronic lymphocytic leukemia.[19,47] In this regard, higher frequency of CD16- NKT-like cells to those NKT-like cells expressing CD16 is expectable. On the other hand, higher frequency of $CD16^-$ NKT-like cells in MB compared to PB may be due to the preferential recruitment of these cells to the endometrium. Such preferential accumulation of $CD16^-$ NKT-like cells in MB was also reflected by higher $CD16^-$: $CD16^+$ NKT-like cells ratio. There is no report on comparative frequency $CD16^{+/-}$ NKT-like cells in endometrium and PB, so comparison of our results with those of other researchers is not possible at the moment. We also observed that women with URSA had higher frequency of PB $CD16^+$ NKT-like cells compared to normal controls, which is supported by previous reports showing that increased frequency of these cells is associated with better IVF outcome[48] but at the same time increase the rate of miscarriage.[49] Indeed, $CD16^+$ NKT-like cells are a major source of many pro-inflammatory mediators that are detrimental for successful pregnancy.[20,21] In both PB and MB, normal fertile women showed higher $CD16^-$: $CD16^+$ NKT-like cells ratio compared to infertile and URSA patients showing that a delicate balance of $CD16^{+/-}$ NKT-like cells is essential for successful implantation and pregnancy.

We next examined cytokine profile of PB and MB NKT-like cells in patients and controls. In overall, the cytokine production pattern by NKT-like cells of all groups showed a similar trend with some exceptions. According to Tsuda et al report, NKT cells were present in first trimester human decidua 8-fold higher than their frequency in PB pointing to their important

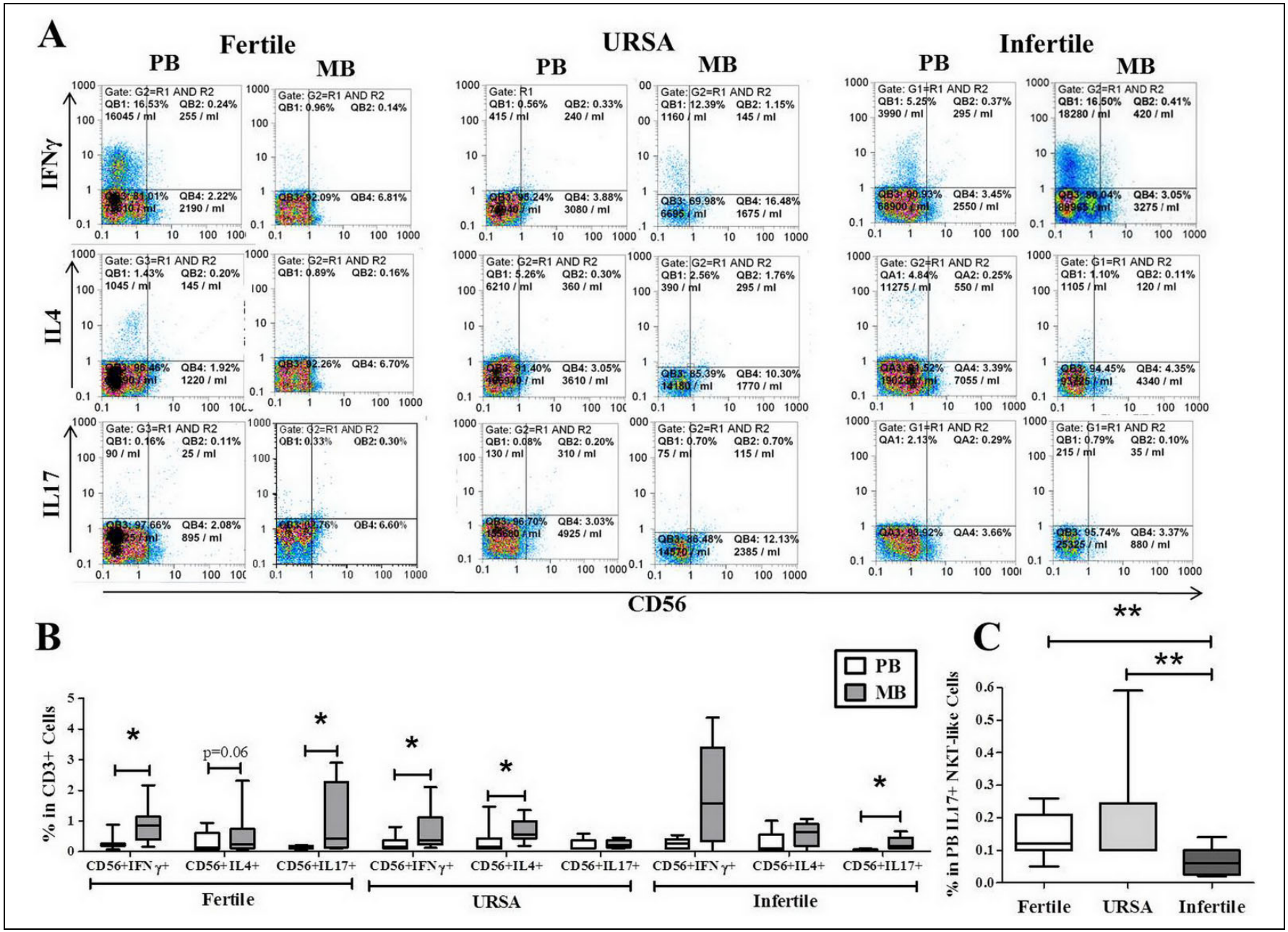

Figure 6. Comparison of Interferon-gamma (IFNγ), interleukin 4 (IL4), and interleukin 17 (IL17)-producing natural killer T (NKT) cells frequency in peripheral blood (PB) and menstrual blood (MB) of fertile, URSA, and unexplained infertile women. A, Representative flow cytometry plots for marker analysis in 3 groups of study. B, Intragroup comparison (n = 8, 9, 9-11, 10, 10 and 5, 5, 5 from left to right), and (C) Intergroup comparison of peripheral blood (n = 11, 13 and 8 from left to right). Frequency of cells was calculated in CD3 gate. Results are shown as box-and-whisker plots (5-95 percentiles). URSA indicates unexplained recurrent spontaneous abortion, *$P \leq .05$; **$P \leq .01$.

role in immunomodulation of feto-maternal interface. This cell type functions in regulation of Th1/Th2 balance by secretion of IFNγ and IL4. Interestingly, the ratios of both IFNγ- and IL4-producing NKT cells were found to be higher in decidua compared to PB. It is noteworthy that they defined NKT cells as $CD3^+CD161^+V\alpha24^+$ cells that are different from the markers we used in the present study. Nonetheless, our results on higher rations of NKT-like cells and IFNγ- and IL4-producing NKT in MB of fertile women are in line with data reported by Tsuda et al.[43] Compared to PB, MB NKT-like cells produced higher amount of pro-inflammatory cytokines, IL17, and IFNγ. Relative predominance of these cytokines in MB versus PB is conceivable considering the inflammatory nature of menstruation. Indeed, it has been reported that IL17 augments extravillous trophoblast invasion.[50,51] On the hand, IL17 may have a role in menstruation through the induction of inflammatory responses mainly by recruitment, activation, induction of neovascularization by the production of pro-angiogenic molecules, and migration of neutrophils and stimulation of production and activity of matrix metalloproteinase 9.[52-54] In line with our previous report on profiling of MB T-cells,[32] we observed that the frequency of IL17-producing NKT-like cells in MB of fertile and infertile women, but not URSA women, is significantly higher than those in PB. This finding may seem contrary to the previous reports showing higher frequency of decidual T17 cells in patients with RSA.[55-59] The exact reason behind this finding is not clear for us, but considering the kinetics of regulatory and counter regulatory immune responses which are mounted successively, our findings on lower frequency of MB $IL17^+$ NKT-like cells in URSA patients during menstruation might be a consequence of higher inflammatory responses in secretory phase. This assumption is further supported by the finding that USRA group was the only group in which MB contained higher frequency of IL4 producing cells compared to PB. Nevertheless, such speculation needs to be confirmed by further experiments. On the other hand, PB of infertile patients contained significantly lower frequency of IL17-producing NKT-like cells compared to controls and URSA patients. Wu et al demonstrated that IL17-producing T-cells are present in decidua and are increased in the PB of clinically normal pregnancies. They showed that these cells support pregnancy by promoting proliferation and invasion and inhibition of apoptosis of human trophoblast cells, suggesting a potential role of IL17 cells in maintaining pregnancy in humans.[59] Decreased frequency of PB $IL17^+$ NKT-like cells in infertile women may be viewed as one possible mechanism leading to immunological infertility.

One limitation of our study was a relatively sample size in each group. This was due to the stringent inclusion and exclusion criteria used for patient selection, unwillingness of some selected patients to be enrolled to the study because of unfavorable mode of MB sample collection, and difficulty in the management of MB sample collection in the second day of menstruation in those patients who were from other cities.

Conclusion

Here, we showed for the first time comparative analysis of NKT-like cells in MB and PB of fertile, infertile, and URSA women. Although PB and MB seemingly have the same histological nature, our results showed that they contained different composition of NKT-like subsets with different cytokine profiles. These results clearly indicate that immune microenvironment of endometrium profoundly affects the cellular composition of MB. To which extend MB immune cells reflect the immune milieu of endometrium is not clear at present and needs further investigations. Nonetheless, our results suggest that MB could be viewed as one potential biological sample for studying immunological imbalances leading to such reproductive complications as infertility and spontaneous abortion.

Finally, the stringent inclusion and exclusion criteria used for patient selection, URSA, and unexplained infertile women, severely constrained us for recruitment of desirable number of patients. Therefore, to consider immunophenotyping of MB immune cells as a useful tool for the investigation of immunological disturbances in pregnancy-related disorders, further investigations are warranted.

Authors' Note

Samira Hosseini performed all experiments, analyzed the data, and wrote the first draft of the manuscript. Soheila Ansari and Mahmood-Jeddi-Tehrani consulted and selected the patients. Jalal Khoshnoodi assisted in doing the experiments. Fazel Shokri and Amir-Hassan Zarnan contributed in study design, conception, interpretation of data, and critically reviewed the manuscript. This study was conducted at Avicenna Research Institute (ARI) and Tehran University of Medical Sciences.

Declaration of Conflicting Interests

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