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REPRODUCTIVE PHYSIOLOGY AND DISEASE

Natural killer cell subsets in endometrial fluid: a pilot study of their association with the endometrial cycle and reproductive parameters

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Abstract

Purpose To investigate if there are natural killer (NK) cells in endometrial fluid (EF) and their relationship with the endometrial cycle and reproductive parameters.

Methods The population under study consisted of 43 women aged 18–40 undergoing infertility workup at our University Hospital in 2021–2022.

The EF samples were obtained at the first visit to our unit, on occasion of the mock embryo transfer. The day of the cycle was considered only in cycles of 27–29 days.

An immunophenotype study of NK in EF was performed by flow cytometry analysis. In a subgroup of women, on the same day, NK was studied in EF and peripheral blood.

Results Our study is the first to evidence NK cells in EF. None of the NK cells observed corresponded to a mature peripheral blood NK cell population (stages 4–5), and neither endometrial nor decidual uNK cells were detected. Nevertheless, we found 2 patient groups with an NK cell subset with a higher expression of CD16+, which could belong to an intermediate or transient stage between the uNK and pbNK NK cell population in the EF. We found that CD16 was significantly increased in the mid-late luteal phase and its correlation with the day of the cycle. The NK immunophenotype was different in EF and peripheral blood.

Conclusion We described a new component of the EF, the NK cells, whose CD16 activity is closely correlated with the day of the cycle. These cells could play a role in implantation/implantation failure.

Keywords CD16 · Endometrial cycle · Endometrial fluid · Implantation · NK cells · Reproductive parameters

Lara Herrera, Myriam Martin-Inaraja, Cristina Eguizabal and Roberto Matorras contributed equally to this work.

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Introduction

Endometrial fluid (EF) is a liquid which has started to receive attention in the last few years. EF is a protein-rich histotroph that contains secretions from the endometrial glands and cleavage products of both the secreted proteins and the glycocalyx (the glycoprotein mucin-rich layer coating the endometrial apical cell surface) [1]. These secretions are derived from two principal sources: a serum transudate arising from the rich capillary plexus surrounding the glands and specific proteins, carbohydrates, and other metabolites synthesized within the glandular cells [2].

Depending on the methodology employed, more than 700 proteins [3, 4], more than 4600 proteins [5], more than 270 lipids [6], and more than 920 micro-RNAs have been detected [7]. Moreover, different proteic, lipidomic, and micro-RNA patterns have been described as prognostic

factors of implantation in the very same cycle in which endometrial fluid was obtained [5-8].

It has been shown that microbiota is present in EF and that some specific microbiota patterns are associated to a favorable outcome in IVF [9]. Moreover, antibacterial proteins have been reported in EF [5], and raw EF has shown antibacterial and antifungal activity [10].

Natural killer (NK) cells are a type of lymphocyte consisting of large granular lymphocytes that belong to the innate immune system. They are derived from hematopoietic progenitor cells (HPCs) in the bone marrow and express the surface marker CD56 [11], and they are defined as $CD3^{-}CD56^{+}$ lymphocytes [12, 13].

Their cytotoxic activity is achieved by the release of the granular components within their cytoplasm (perforin, granzymes) or the secretion of cytokines (tumor necrosis factor-alpha, interleukin (IL)-10, interferon-gamma and transforming growth factor-beta) [14].

There are two main types of NK cells: (i) peripheral blood NK cells (pbNK), which make up the majority of NK cells in human blood, with low CD56 expression (CD56dim) and high levels of Fc receptor for immunoglobulin G (IgG; FcγR)III (CD16), representing about 10–15% of the total lymphocytes in blood and whose activity is predominantly cytotoxic [15]; and (ii) uterine NK cells (uNK), which are mostly $CD56^{bright}$ $CD16^{-}$ cells and which correlate with a more cytolytic phenotype than peripheral NK cells [15, 16] and display a more immune-regulatory role [15].

Peripheral blood NK cells originate from CD34+ hematopoietic progenitors. Before reaching a mature stage, they acquire different surface markers progressively and in a certain order and can be classified into stage 1 (CD34+, CD45RA+, CD117−, CD94−, CD56−, CD16−), stage 2 (CD34+, CD45RA+, CD117+, CD94−, CD56−, CD16−), and stage 3 (CD34− CD117+, CD94−, CD56−, CD16−). Once they reach a mature stage, NK cells are phenotypically described by their surface markers as stage 4 (CD34−, CD94+, CD117+/−, CD56bright, CD16+/−) and stage 5 (CD34−, CD94+/−, CD117−, CD56dim, CD16+) [12]. However, there is no data available regarding the maturity stages of the uNK cells.

Peripheral blood NK cells (stage 5) have been demonstrated to show significant cytotoxic activity with well-established antiviral and anti-neoplastic functions, while uNK cells have little cytotoxic activity but are a rich source of cytokines, particularly angiogenic ones, with possible roles in the regulation of trophoblast invasion and angiogenesis [17, 18]. Furthermore, peripheral blood NK cells may not reflect the condition of the endometrium where implantation occurs. In contrast, large numbers of uNK cells appear in the mid-secretory phase, although the mechanism is still not known. There are two theories concerning the origin of uNK cells: (i) recruitment from pbNK cells which subsequently differentiate in the uterine microenvironment into the uNK cell phenotype through a series of organized processes; or (ii) that uNK cells come from the in utero proliferation and differentiation of stem cells or endogenous NK cells in the endometrium [17, 19]. The former theory is the rationale for testing pbNK cells, although the latter is the more widely held view. In contrast to pbNK cells, uNK cells are resident in the endometrium and constitute 70% of endometrial leucocytes, the most predominant leucocyte population during the time of implantation and early pregnancy [20, 21].

NK cells have been thought to be associated with implantation failure (IF), recurrent miscarriage (RM), or infertility due to either NK cell cytotoxicity or receptor/gene expression, but results are controversial [14, 22]. There are conflicting reports regarding the changes in peripheral NK cell activity during the endometrial cycle [23-27]. On the other hand, uNK are present in the proliferative phase, but they increase in number substantially in the mid-secretory phase and represent the major endometrial lymphocyte population in the late secretory phase and the first trimester of pregnancy [21-28].

The aim of our study is to investigate the presence of NK cells in endometrial fluid and their immunophenotype, to ascertain their changes, if any, during the endometrial cycle, and to determine their association with reproductive parameters.

Material and methods

Study population

The population under study consisted of 43 women undergoing infertility workup at the Reproduction Unit of our University hospital.

The inclusion criteria were (a) age 18–40 and any one of the following: (b1) infertility duration >1 year, (b2) single women with reproductive desire, and (b3) oocyte vitrification for medical reasons. The exclusion criteria were infectious diseases, abnormal uterine cavity on ultrasound, myomas, tubal factor, abnormal karyotype, immunological or metabolic diseases, and BMI > 36.

The main characteristics of the population are depicted in Table 1. The median age was 37.0 years [34.0; 38.0], and the median body mass index (BMI) was 24.9 [22.6; 28.7]. The main infertility diagnoses were idiopathic infertility (46.5%), male factor (18.6%), endometriosis (7.0%), and single woman (7.0%). Five women had previously undergone artificial insemination, without pregnancy. None had previously undergone IVF. 20.9% of women had previous children, 23.3% had previous spontaneous miscarriages, and 16.3% had elective abortions.

Table 1 Demographic characteristics

	$N = 43$
Infertility duration (years)	2.6 ± 1.3
Age (years) [25th;75th]	37.0 [34.0; 38.0]
BMI (kg/m^2) [25th;75th]	24.9 [22.6;28.7]
Smoking (%)	27.9 (12)
Day of the cycle	14.8 ± 5.9
Previous pregnancy (%)	53.5 (23)
Previous children (%)	20.9 (9)
Previous miscarriage and/or elective abortion (%)	37.2 (16)
Previous spontaneous miscarriage (%)	23.3 (10)
Elective abortion (%)	16.3 (7)
Day of the cycle (%)	
0–12	38.5 (15)
13–18	33.3 (13)
≥ 19	28.2 (11)
NA	(4)*
BMI (categorized) (%)	
18–25	51.2 (22)
26–30	27.9 (12)
>30	20.9 (9)
Main infertility diagnosis (%)	
Unknown factor	46.5 (20)
Male factor	18.6 (8)
Single woman	7.0 (3)
Endometriosis	7.0 (3)
Oocyte cryopreservation	2.3 (1)
Other	18.6 (8)

Values are expressed as mean ± standard deviation unless otherwise expressed. Numbers inside parentheses correspond to the number of cases

* = 2 samples corresponded to disovulatory cycles and in 2 the day of the cycle was not registered

The samples were obtained on the first visit to our unit, on occasion of the mock embryo transfer which is systematically performed during the infertility workup in patients where IVF or IUI is considered. In our study, additionally, we performed immediately before EF aspiration a vaginal ultrasound in order to confirm that endometrial and ovarian findings were consistent with the reported day of the cycle. Our study protocol foresaw the rejection of cases with inconsistency between chronological and ultrasound dating, but no case was excluded for this reason.

The day of the cycle was considered only when the cycle length was 27–29 days.

Table 2 Median values of CD94, CD117, CD56, and CD16

CD94	1.77 [0.60; 9.65]	43
CD117	2.20 [0.51; 8.91]	43
CD56	6.59 [2.92; 17.40]	43
CD16	5.98 [1.65; 36.0]	43

Endometrial fluid sample collection

Endometrial fluid was obtained by manual aspiration with a 10 mL syringe connected to an "embryo transfer catheter" (Frydman catheter, CCD Laboratories, Paris, France) as previously reported [8, 29]. Aspiration was performed under ultrasound control gently, in order to avoid traumatizing the endometrium. Samples obtained were expelled into a Falcon tube without any medium, and the distal 3–4 cm of the catheter were sectioned (in two or three fragments) and were placed also in the same Falcon tube. Samples were kept at 4 °C and were processed in the following 3 h. Aspirate volumes ranged from 5 to 50 µl. Blood-tainted samples were excluded from the study.

Endometrial fluid processing

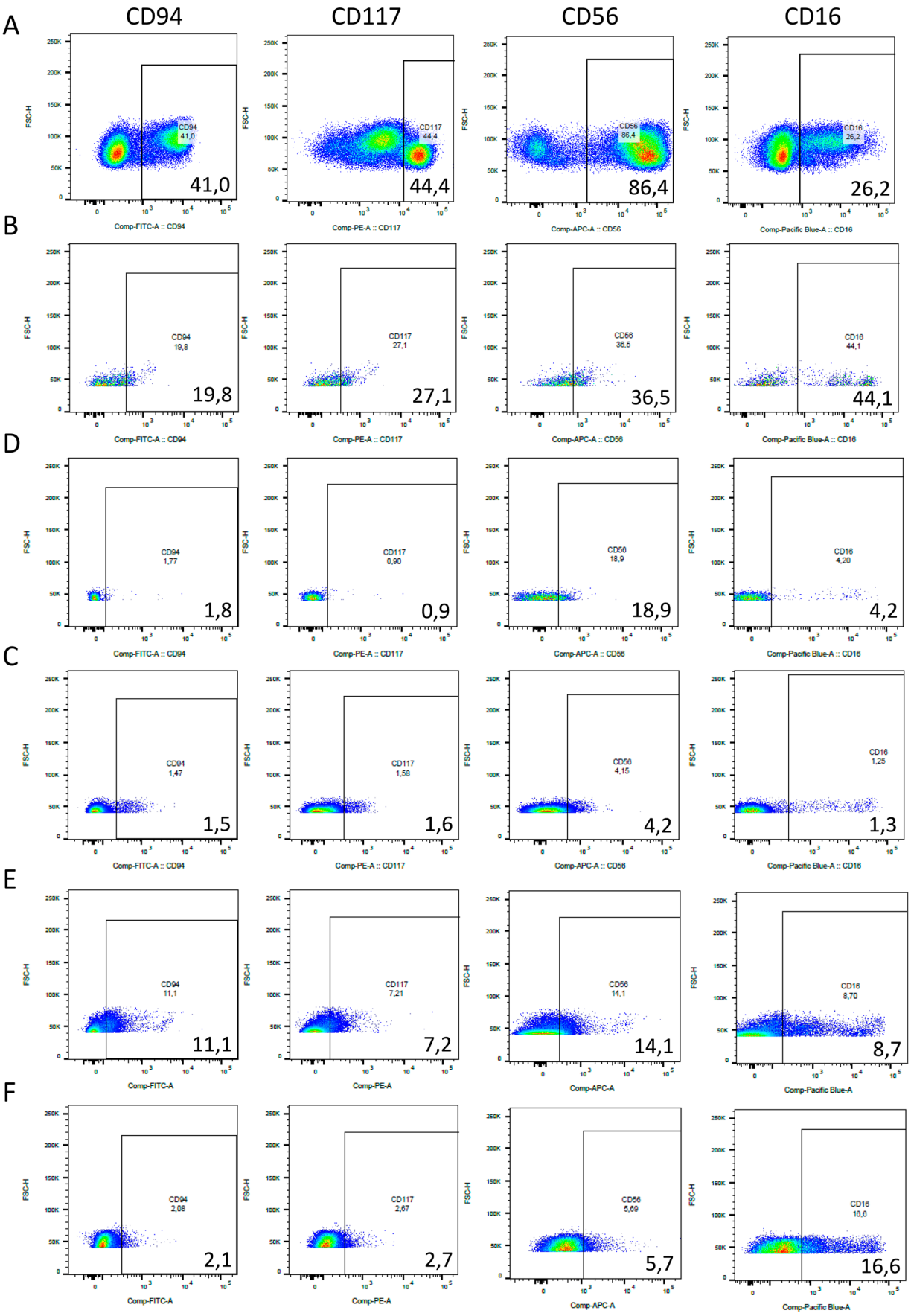
In order to recover the EF and the cells within it, we passed 500 ul of PBS + 10% FBS through the fragments of the catheter and poured it into the original Falcon tube where the aspirate sample had been left. Cells were collected in 15 ml Falcon tubes and filtered with a 40-µm nylon cell strainer in order to prevent any clumps or residual tissue. Then, the cell samples were passed to cytometry tubes. Depending on the sample, between 10,000 and 100,000 cells were recovered.

Flow cytometry analysis

Cells were washed with PBS+10% FBS and incubated for 30 min at 4 °C for labeling with anti-CD94-FITC (BD Biosciences, clone HP-3D9), anti-CD117-PE (Miltenyi Biotec, clone A3C6E2), anti-CD3 PerCP Cy5.5 (Biolegend, clone SK7), anti-CD56-APC (Biolegend, clone MEM-188), and anti-CD16-BV421 (BD Biosciences, clone 3G8) in order to determine the NK cell population. 10,000–30,000 events were acquired for analysis. Flow cytometry cell populations were analyzed using FlowJo v.X.0.7 (TreeStar Inc.). Antibody expressions are represented as percentage mean (standard deviation) and those without a normal distribution with the median (interquartile range).

Blood sample collection and processing

Blood samples from a subset of 8 women were collected in an EDTA 10-ml tube, a few minutes after endometrial fluid aspirate. Samples were studied at the Basque Biobank (http://www.biobancovasco.org) under an institutional review board-approved protocol by the Basque Committee of Ethics and Clinical Research (ref: 2014138). A total of 200 µl of blood were transferred on top of the previously mentioned antibodies in a cytometer tube and incubated for 30' in the dark at RT. Then, in order to remove red blood


A
CD94
CD117
CD56
CD16
41,0
44,4
86,4
26,2
B
19,8
27,1
36,5
44,1
D
1,8
0,9
18,9
4,2
C
1,5
1,6
4,2
1,3
E
11,1
7,2
14,1
8,7
F
2,1
2,7
5,7
16,6
FSC-H
Comp-FITC-A :: CD94
Comp-PE-A :: CD117
Comp-APC-A :: CD56
Comp-Pacific Blue-A :: CD16
Comp-FITC-A
Comp-PE-A
Comp-APC-A
Comp-Pacific Blue-A

◂**Fig. 1** Immunophenotyping assay of NK cells in EF. Markers used CD94, CD117, CD56, and CD16. **A** Control group: peripheral blood NK cells; **B** Group 1: high expression of CD56, CD16, CD117, and CD94 markers in low number of cells; **C** Group 2: higher expression of CD56 in comparison with the expression of rest of markers; **D** Group 3: low and uniform expression of CD56, CD16, CD117, and CD94; **E** Group 4: high expression of CD56, CD16, CD117, and CD94 markers in high number of cells; and **F** Group 5: higher expression of CD16 in comparison with the expression of rest of markers

cells from the sample, red blood cell lysis buffer (Roche) was used according to the manufacturer's instructions. Briefly, 2 ml of the buffer were added and incubated for 15' at RT. Cells were washed twice with PBS at 500 g for 5' at RT. Finally, cells were resuspended in 400 µl of PBS.

Data and statistical analysis

We performed the normality test for continuous variables using the Shapiro-Wilks test. Variables following a normal distribution were presented with the mean (standard deviation) and those without a normal distribution with the median (interquartile range). Differences between variables were analyzed with the Chi-square test for categorical variables and Student's *t*-test or Mann-Whitney *U*-test, ANOVA, or Kruskal-Wallis test for continuous variables. In multiple comparisons, the Bonferroni correction was employed. Linear regression models were performed for the association of continuous variables with the NK cells. Scatter plots were also presented. All analyses were performed with the statistical software R (version 4.1.1): A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Results

Immunophenotype characterization of NK cell population in endometrial fluid

Depending on the surface marker pattern reported in peripheral blood and uterine NK cells, key markers (CD56, CD16, CD117, and CD94) were selected in order to determine the NK cell immunophenotype in the EF [30]. We analyzed all samples from 43 women undergoing an infertility workup at the Reproduction Unit of our University Hospital. Firstly, we analyzed the mean expression of the chosen NK cell markers in all the samples studied (Table 2). In general, we found expression of NK cell markers in all the samples. However, we decided to subdivide them into five different study groups in order to observe any difference in the NK cell expression pattern compared with the peripheral blood NK pattern. These are the criteria of the five groups studied: (1) high expression of CD56, CD16, CD117, and CD94 markers in a low number of cells ($n = 6$); (2) higher expression of CD56 in comparison with the expression of rest of the markers ($n = 10$); (3) low, uniform expression of CD56, CD16, CD117, and CD94 ($n = 10$); (4) high expression of CD56, CD16, CD117, and CD94 markers in a high number of cells ($n = 5$); and (5) higher expression of CD16 in comparison with the expression of rest of the markers ($n = 13$) (Fig. 1).

Secondly, we observed that the pattern found in groups 2, 3, and 4 is more similar to the expression pattern of peripheral blood NK cells. There was a high expression of CD56, a low expression of CD117 and CD94, and a lower expression of the CD16 marker compared to CD56. By contrast, in groups 1 and 5, we observed that the expression of CD16 was higher than CD56 expression and CD94 and CD117 was higher, especially in group 1, than the samples from groups 2, 3, and 4.

In addition, NK cells from EF were compared to the peripheral blood NK cells from the same woman ($n = 8$). The observed patterns were different, suggesting that NK cells detected in the EF were different from those from peripheral blood (Fig. 2).

NK cell markers and clinical parameters

In terms of demographic parameters (Table 3), CD values were not influenced by the woman's age, BMI, or smoking. NK cell surface markers were not associated with previous reproductive history (Table 3).

The CD16 expression was much higher in the mid-late secretory phase (48.7 [21.5; 82.9]) than in the early-mid follicular phase (3.93 [1.60; 15.1]) and in the peri-ovulatory phase (4.20 [2.30; 33.2]) (Table 4). There was a statistically significant association between the day of the cycle and CD16 expression (2.409; 95% CI: =1.051–3.766), *p*-value = 0.001) (Fig. 3).

The other CD markers did not change during the endometrial cycle.

Discussion

The role of EF in reproduction is not well understood. Apart from providing nutrients to the pre/peri-implantation embryo, it has been suggested that it could play other roles in promoting implantation [4, 6-8]. Thus, over 270 lipids [6] and over 4600 proteins [5] have been found in EF, a number of them with antibacterial activity, or related with immune defense, immune innate defense, and inflammation [4, 5, 8]. Moreover, a number of microorganisms have been described in EF [9, 31] as well as micro-RNA and exosomes [7, 32]. EF has shown antibacterial and antifungal activity in vitro [10].

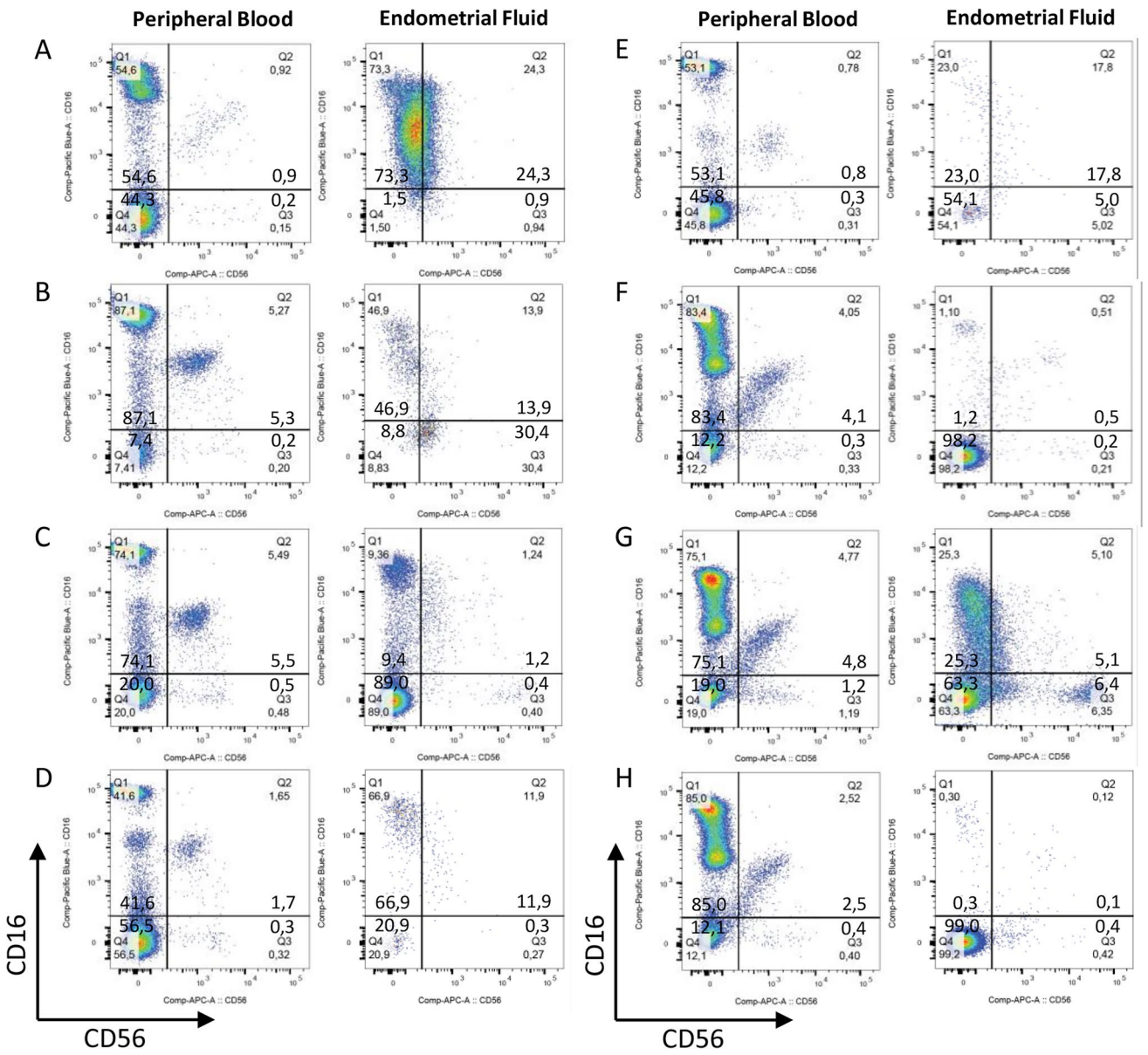


Fig. 2 CD16 and CD56 expression in peripheral blood and endometrial fluid NK cells. Comparison of NK cell population from whole blood and endometrial fluid from 8 different women (**A**–**H**). The percentage of the expression of CD56 and CD16 markers was analyzed to check differences in population patterns

NK cells can be classified into peripheral NK cells and uterine NK cells. Uterine NK cells are less cytotoxic than peripheral NK cells [33, 34] and produce factors that mediate placental implantation and tissue remodeling, especially in spiral artery remodeling [35, 36]. As far as we know, our study is the first to investigate NK cells in EF, and in it, we observed NK in all the samples analyzed, independently of the day of the cycle or the presence of small amounts of blood tainting in the samples. In all the cases, the NK cells expressed CD56, CD94, CD117, and CD16 markers, although the expression pattern differs from the peripheral blood NK cells expression pattern. Moreover, differences among patient groups have been detected regarding the expression pattern. A specific NK pattern was identified, consisting of the highly expressed CD16 marker in comparison with the CD56 marker. This pattern correlated with no specific demographic or infertility parameter. We have to highlight that at no point were uterine NK cells detected, suggesting that uterine NK cells did not leave the endometrium at any moment in the endometrial cycle. It has been reported that peripheral NK cell numbers and activity can fluctuate depending on the phase of the menstrual cycle [37], time of day [38], parity [39], and sympathetic response to stressors [40], as well as the measurement method [14, 41,

Table 3 CD94, CD117, CD56, and CD16 values and smoking, age, and body mass index (BMI). CD94, CD117, CD56, and CD16 values, clinical parameters, and reproductive history

		CD94	CD117	CD56	CD16
Smoking	Yes ($n = 12$)	1.59 [0.67;5.92]	2.03 [0.47;5.90]	6.10 [3.04;13.50]	7.04 [1.90;45.0]
	No ($n = 31$)	1.80 [0.55;9.80]	2.30 [0.54;10.10]	6.59 [2.92;17.40]	4.20 [1.65;30.50]
Age	<35 ($n = 12$)	2.54 [0.67;3.78]	2.25 [0.47;5.90]	5.94 [3.89;9.0]	3.27 [2.05;26.0]
	35–38 ($n = 24$)	1.65 [0.50;12.60]	2.03 [0.43;9.60]	6.30 [2.68;18.30]	7.14 [1.83;35.50]
	> 38 ($n = 7$)	1.80 [1.05;7.88]	7.21 [0.60;11.70]	7.69 [4.96;16.10]	8.70 [1.65;39.10]
BMI	18–25 ($n = 22$)	2.60 [0.62;11.60]	2.65 [0.54;9.00]	8.25 [3.14;18.10]	8.50 [1.45;42.0]
	26–30 ($n = 12$)	2.60 [1.38;7.83]	3.45 [0.82;10.60]	4.96 [3.65;17.30]	7.04 [2.07;25.90]
	>30 ($n = 9$)	0.75 [0.50;0.90]	0.61 [0.23;2.67]	5.36 [0.80;6.92]	2.60 [2.10;16.60]
Previous pregnancy	Yes ($n = 23$)	1.14 [0.60;10.90]	3.10 [0.54;10.10]	6.92 [1.84;13.10]	8.70 [2.20;38.60]
	No ($n = 20$)	1.79 [0.62;4.39]	2.03 [0.45;8.44]	5.97 [3.89;18.0]	4.07 [0.98;25.90]
Previous spontaneous miscarriages	Yes ($n = 10$)	1: 0.90 [0.55;19.40]	1: 11.0 [0.25;14.70]	1: 18.1 [2.90;28.0]	1: 20.0 [1.70;38.60]
		≥2: 8.50 [4.45;9.65]	≥2: 5.0 [2.55;10.60]	≥2: 6.92 [3.61;7.46]	≥2: 71.8 [37.2;82.9]
	No ($n = 33$)	1.77 [0.69;4.30]	2.11 [0.58;8.38]	5.69 [3.03;14.10]	4.20 [1.39;27.80]

Expressed as median [interquartile range]

No significant differences

42]. Although the study of antibody-dependent cellular cytotoxicity could be of interest, the small number of recovered cells precluded its analysis.

It is well known that age, obesity, and smoking have a negative impact on female fertility [43], acting through different mechanisms. The CD NK cell markers in EF were not influenced by the woman's age, which is in agreement with the lack of influence of maternal age on endometrial receptivity evidenced by pregnancy rates in oocyte donation programs [44, 45]. Smoking and obesity also did not influence NK cell activity.

The endometrium experiences a number of cyclical changes, which were described in the pivotal work of Noyes et al. in 1950 [46]. From a physiological point of view, the objective of these changes is to prepare the endometrium for implantation and early embryo development. We have to highlight that CD16 expression in EF was much higher in the secretory phase and its values increased with the day of the cycle. The other CD markers did not change during the endometrial cycle. Therefore, it could be speculated that CD16 expression may play a role in implantation.

Table 4 CD94, CD117, CD56, and CD16 values and day of the cycle

	Day of the cycle ($n = 15;13;11$)			
	0–12	13–18	≥19	*p*
CD94	2.08 [0.68;10.30]	1.77 [0.40;11.10]	1.80 [0.93;9.65]	0.84
CD117	2.67 [0.45;6.66]	1.90 [0.23;11.0]	8.38 [2.03;9.12]	0.50
CD56	5.69 [3.59;17.40]	12.0 [4.62;18.10]	5.29 [2.15;10.0]	0.33
CD16	3.93 [1.60;15.10]	4.20 [2.30;33.20]	48.7 [21.50;82.90]	0.02

Expressed as median [interquartile range]

Previous reports on peripheral NK activity during the menstrual cycle are conflicting. Lower peripheral NK cell activity has been reported during the follicular phase [23], the early follicular phase [47], the peri-ovulatory period [37], and the luteal phase [48], whereas some have reported no differences [24] or no differences in NK cell activity but an increase during the peri-ovulatory phase in the number of NK cells per milliliter of blood [27]. Furthermore, uNK cells, which are already present in the proliferative phase, increase remarkably in the mid-secretory phase and are the

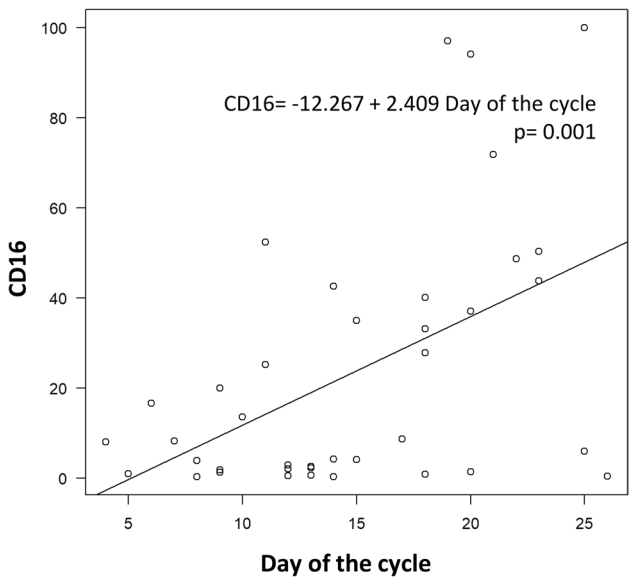


Fig. 3 Association between the day of the cycle and CD16 expression

major lymphocyte population in the late secretory phase [21, 28, 49]. Thus, our findings concerning CD16 activity in EF during the endometrial cycle display a pattern which is similar with that reported in uNK cells.

The pattern of CD expression markers we found was not influenced by previous miscarriages. It is well known that miscarriage has many different causes, maternal age being one of the most frequent [50]. There is controversy regarding the role of NK cells in idiopathic recurrent miscarriage and/or implantation failure. Some studies have reported a significantly higher percentage of NK cells compared with healthy individuals [49, 51]. However, a number of meta-analyses have not found a clear association between NK cells and recurrent miscarriage/implantation failure [14, 52] nor with the efficacy of immunotherapy in RM [53, 54]. In our study, NK markers were similar in women with and without previous miscarriages. However, it has to be highlighted that the population we studied consisted of women with 1–2 previous spontaneous miscarriages and not of RM.

There is also controversy regarding which marker is better associated with idiopathic RM/IF: pbNK or uNK. It has been suggested that uNK cells would be of more interest since they intervene in the placentation process.

In our study, none of the NK cells observed corresponded to mature peripheral blood NK cell populations (stages 4–5) [12], and neither endometrial nor decidual uterine NK cells were detected at any point. Nevertheless, we found 2 patient groups with an NK cell subset showing a higher expression of CD16+ that did not correspond to the peripheral blood phenotype, which could belong to an intermediate or transient stage between the uNK and pbNK NK cell populations in the EF [13, 30]. From our study, it can be understood that in the peri-implantation process, EF NK cells would be of more importance since they are present in the EF, which includes the pre-implantatory embryo.

CD16 is a low-affinity Fc gamma receptor for IgG binding and is expressed on NK cells to trigger cytotoxicity or cytokine secretion in naive NK cells. It has been described that CD16 surface expression is correlated with NK-cell maturation and that pbNK cells can acquire CD16 at a later stage in their development, in association with a dramatic gain in IFN-γ expression [55]. This evidence suggests that the CD16 features could be an adaptive outcome of pbNK/dNK cells responding to unique environmental stimulation during decidualization and placentation, both of which are driven by local and systemic signals in human pregnancy. Traditionally, $CD56^{bright}$CD16- NK cells were considered to be prominent cytokine producers with little cytotoxicity, whereas $CD56^{dim}$CD16+ NK cells, due to their content of high quantities of cytotoxic granules, were efficient killers but poor cytokine producers. However, increasing evidence suggests that in some tissue-specific microenvironments, CD16+ NK cells are a potent source of proinflammatory cytokines and chemokines, produced upon target cell recognition. Since the activation of the CD16 signal can stimulate the expression of many cytokines, via calcium-dependent mechanisms, the presence of the CD16+ dNK subpopulation could be critical to complementing maternal immune response and promoting human pregnancy. This would lead to a subsequent impact on the immunological regulation of decidual resident NK subsets in normal and abnormal pregnancies [56].

We would like to highlight some points from our study. Firstly, NK CD16 in EF increases during the endometrial cycle, suggesting it plays a role in implantation. Secondly, EF NK cells probably play a more relevant part in the implantation mechanism than uterine NK cells, since the pre-implantatory embryo is in the EF. NK cells have a pattern of surface marker pattern more similar to peripheral blood NK cells than uterine NK cells.

However, it is important to remember, that besides containing NK cells, the EF is rich in peptides, lipids, exosomes, micro-RNA, and microorganisms, which could mean that there is a balance and crosstalk between them. It seems likely that the relationship between immune mechanism and implantation is related to the crosstalk rather than to a single NK cellular surface marker.

Our study opens the possibility of a new field of translational research for some processes whose pathophysiology and/or treatment have many unknown aspects, such as implantation, IF, RM, and, in general, reproductive immunology.

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Declarations

Ethical approval All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. The study was approved by our Institutional Ethical and Investigation Board (CEIC code E21/63).

Informed consent Written informed consent was given to women and, in the case of couples, also to their partners.

Conflict of interest The authors declare no competing interests.

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