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Original Article

Evaluation of peripheral blood NK cell subsets and cytokines in unexplained recurrent miscarriage

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Abstract

Background: Recurrent miscarriage is considered as one of the main problems in women's reproductive health. The aim of the present study was to evaluate the natural killer cells (NK cells) and cytokines in unexplained recurrent miscarriage and fertile women.
Methods: In this case−control study, 40 women with unexplained recurrent miscarriage were assigned to the case group and 40 fertile women were assigned to the control group. NK cell subsets ($CD56^+$ $CD16^+$/$CD56^+$ $CD16^-$) and cytokines (IL-2/IL-12) levels in the peripheral blood (PB) were used for assessing immunologic problems. The percentage of peripheral blood NK cells ($CD56^{dim/bright}$) was identified by flow cytometry.
Results: The obtained results showed a significant difference in $CD56^+$ $CD16^+$ and $CD56^+$ $CD16^-$ between the two groups. Also, there was no significant difference in the IL-2 and IL-12 between the two groups. A cut-off value of ≥5.25% ($p < 0.001$) and ≥3.4% ($p < 0.015$) for the increased percentage of $CD56^+$ $CD16^+$ and $CD56^+$ $CD16^-$cells in the PB become predictive of recurrent miscarriage.
Conclusion: Increased NK cells in the PB of women with recurrent miscarriage strongly establish prospective researches to recognize the predictive value of these parameters in the evaluation of patients with recurrent miscarriage.
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Keywords: Cytokines; Natural killer cells; Peripheral blood; Recurrent miscarriage

1. Introduction

Infertility and recurrent miscarriage are two main problems for women in the reproductive age.[1,2] Pregnancy is a challenging condition that fetus has direct contact with the mother, but does not reject.[3] Recurrent miscarriage refers to the loss of two or more pregnancies before 20 weeks of gestation.[2,4] It affects 1−5% of couples of reproductive age[5] and occurs due to genetic or uterine problems, thrombophilia, autoimmune and endocrine diseases, infections, and several environmental factors[6] but it is not determined in 50%.[7]

NK cell function is very important for reproductive success. These cells make up 5−10% of peripheral blood lymphocytes and 70−90% of uterine lymphocytes which are categorized to $CD16^+$ $CD56^{dim}$ and $CD16^-$ $CD56^{bright}$ with surface markers.[8,9] More NK cells in the peripheral blood are $CD16^+$ $CD56^{dim}$, but in the endometrium are kind of $CD16^-CD56^{bright}$. It is reported that peripheral NK cells ($CD56^{dim}$) have an important cytotoxic function, but uterine NK cells (uNK cells) produce cytokines.[10] It proved an

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increment in endometrial and peripheral blood NK cells in the recurrent miscarriage,[11] and *in vitro* fertilization (IVF).[12] Some studies have reported an association between uNK cells and infertility,[13] other studies showed the opposite result.[14] Also, some studies have indicated that uNK cells may have a useful result on reproductive outcomes.[15] It is reported that measurement of uNK cells in nonpregnant women with a history of recurrent abortion can not predict the pregnancy result.[16] In the normal pregnancy, there is a balance increase of Th2 and decrease of Th1 cytokines, and an increase of Th1 may be a cause of pregnancy failure.[17] NK cell activity in destroying tumor cells increase due to tumor necrosis factor (TNF), interferon (IFNs), IL-2 and IL-12 (Th1 cytokines).[18]

Immunological disorders have been suggested to play a main role in recurrent miscarriage. Many factors are known to correlate with recurrent miscarriage, and it is indeed tough to work them out. Although, NK cells and T cells are a part of the cause of abortion.

We hypothesized that the levels of NK cell subsets (CD56 and CD16), and cytokines (IL-2 and IL-12) in PB would be different in patients with unexplained recurrent miscarriage compared with healthy controls. Therefore, considering the important role of the immune system in recurrent miscarriage, the present study was conducted to compare the levels of peripheral NK cells and cytokines between women with history of unexplained recurrent miscarriage and fertile women, as well as to determine the correlation between NK cells and cytokines (Th1) in North of Iran.

2. Methods

2.1. Study design and population

This case–control study was conducted from May 2015 to August 2016 in Babol, Iran. 80 people were examined in this study. In total, 115 women with recurrent miscarriage were referred to the Research Center for Infertility of Babol University of Medical Sciences. The recurrent miscarriage was defined as having had two or more consecutive abortions in the first trimester of pregnancy. Out of those referred, 75 women were excluded on the inclusion criteria, and the final case sample comprised 40 women with unexplained recurrent miscarriage. All women with known probable etiologies for recurrent miscarriage were excluded from the study. The inclusion criteria for the recurrent miscarriage patients included having experienced at least two consecutive idiopathic abortions of a desired pregnancy with a sexual partner; regular menstruation; no history of polycystic ovary syndrome (PCOS); normal gynecological status, anatomy, and karyotype; normal levels of the antiphospholipid antibody, anti-nuclear antibody, anticardiolipin antibody, antithrombin III, lupus anti-coagulant, homocysteine, protein S, protein C, factor V Leiden, anti-thyroid peroxidase (Anti-TPO), thyroid hormones, and prolactin; and normal spermogram and karyotype of the sexual partner. Women without recurrent miscarriage, who were referred to primary health care centers (PHCs), were selected as control subjects. These 135 healthy, nonpregnant women with at least one living child did not have a history of infertility, previous abortion, preterm deliveries, or stillbirths. Among them, 95 women were excluded from the study due to dissatisfaction for testing, and finally, a sample of 40 women was used as the control group. The case and control groups were evaluated from three months to one year after abortion or childbirth, respectively.

2.2. Isolation of peripheral blood mononuclear cells (PBMCs)

Five milliliters of peripheral blood samples from all women of the large antecubital vein was taken into EDTA-coated tubes during the follicular phases of the menstrual cycle at 8 AM. Fresh PBMCs were isolated from venous blood samples using density gradient centrifugation (BAG Germany) at 1000 × *g*, at room temperature for 5 min. After double washing of isolated PBMCs with Hanks' balanced salt solution (HBSS), an ultimate concentration of 5×10^6 cells/ml in phosphate-buffered saline (PBS) completed with 5% fetal bovine serum (FBS, Gibco) by centrifugation (Hettich, Germany, 500 × *g*, at room temperature for 5 min). Collected PBMCs were stored at 4 °C in the dark.

2.3. Flow cytometry

In this study, flow cytometry was performed in Razi laboratory, Babol, Iran, and we used a Partec flow cytometer (Nuremberg, Germany). Cell staining for flow cytometry analysis was conducted with predetermined condensations of the following mouse anti-human fluorochrome-conjugated monoclonal antibodies (mAb) includes: CD45 perCP (perCP, Dako, Denmark, Cat number: PR701), CD16-fluorescein isothiocyanate (FITC, DAKO, Denmark, Cat number: F 7011), and $CD56^-$ phycoerythrin (PE, DAKO, Denmark, Cat number: R 7127). Isotype controls included mouse IgG1-FITC (DAKO, Denmark, Cat number: XO 929, XO 927), IgG1-PE (DAKO, Denmark, Cat number: XO 930, XO 928), and IgG1-perCP. Lymphocytes were incubated with the above markers at 4 °C for 30 min. 100 µl cell suspension (that include 5×10^6 cells/ml) were taken and stained with suitable fluorochrome conjugated mAbs for 30 min at 4 °C in the dark until flowcytometric analysis. Flomax software (Partec) was used for data analysis. Since some NK cell subsets constitute a small percentage of the cells, approximately 20,000 cells were analyzed in the gate of R1 ($CD45^+$ lymphocyte cells) for each sample. Gating on the lymphocyte population could not be easily and precisely performed especially based on a 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 analysis and was performed on this gate. NK cell subsets were quantified based on the percentage of triple markers. Representative

flowcytometry dot plots illustrating the analysis method for the detection and enumeration of NK cell subsets are shown in Fig. 1.

2.4. Cytokine assay

Serum was obtained from fresh blood samples, and it was frozen at −80 °C until processed. The serum concentration of IL-2 and IL-12 was determined by commercially available highly sensitive sandwich ELISA kits from Bender Med system GmbH, (Affymetrix and eBioscience, Vienna, Austria) according to the manufacturer's instructions in the laboratory of Cellular and Molecular Research Center, Babol University of Medical Sciences, Iran. The results were determined using a microplate reader (RT-2100C, Rayto, China). It should be noted that the sensitivities of

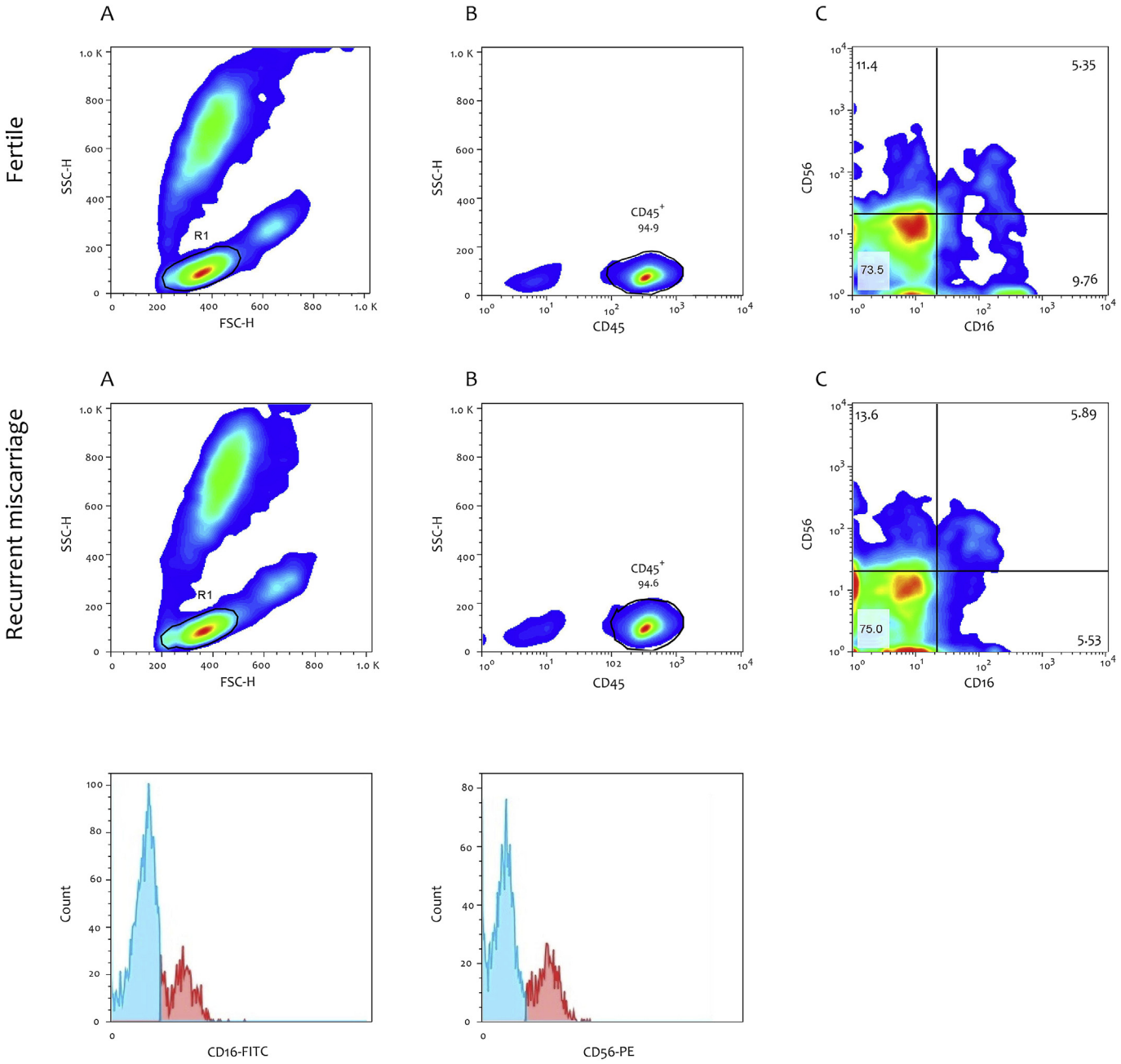

Fig. 1. Flow cytometric (pseudo-color/smooth) plots showing the results for the identification of NK cells in peripheral blood of a fertile and recurrent miscarriage sample using three-color staining. (A) Lymphocytes were gated based on size and granularity using FSC vs SSC. The R1 gate was used to select lymphocytes for analysis. (B) Exclusion of CD45 positive cells was sub-gated using PerCP anti-human CD45 antibody. (C) NK cells were identified with FITC anti-human CD16 antibody and PE anti-human CD56 antibody staining. The numbers in the upper left, lower right, and upper right quadrants in the smooth plot represent the percentages of $CD56^{+}CD16^{-}$, $CD56^{-}CD16+$, and $CD56^{+}CD16^{+}$ cells, respectively. In addition, histogram plots showed the flow cytometric results for the $CD16^{-}$FITC and $CD56^{-}$PE along with isotype control.

each assay were as follows: IL-2, 9.1 pg/mL, and IL-12, 2.1 pg/ml. All results were multiplied in 2 on the manufacturer's instructions.

2.5. Ethical considerations

The Ethics Committee of the Babol University of Medical Sciences approved the study (ID: MUBABOL.REC.2015.42). The participants signed a written informed consent form prior to the participation in the study, in keeping with the recommendations of the Declaration of Helsinki.

2.6. Statistical analysis

Data were analyzed using the Statistical Package for the Social Science (SPSS) 22.0 software. The differences of the subset of natural killer cells and cytokines levels between two groups were analyzed using independent samples *t*-test and nonparametric Mann–Whitney U test. Pearson's correlation was utilized to analyze the correlations between variables. A receiver operating characteristic (ROC) curve was applied for RPL and control groups. A *p*-value less than .05 was considered significant.

3. Results

The demographic and reproductive characteristics of the participants showed that there was no significant difference between the recurrent miscarriage and fertile groups regarding age and body mass index. The mean of living child in control group and abortion in the case group was 1.45 ± 0.50 and 2.75 ± 1.01, respectively ($p = 0.0001$). Fig. 2 shows the findings related to NK cell subsets in the two groups. There was a significant difference in $CD56^+$ $CD16^+$ and $CD56^+$ $CD16^-$ between the two groups. Table 1 shows the findings related to cytokine levels in the two groups. Based on the Mann–Whitney U test, there was no significant difference in the IL-2 and IL-12 between the two groups. Besides, Pearson correlation coefficient showed a significant association between two cytokines ($p = 0.0001$, r = 0.724). Receiver operating characteristic (ROC) curves were plotted to compare the percentages of NK cells in PB samples of the recurrent miscarriage and fertile groups (Fig. 3). The area under the curve (AUC) for peripheral NK cells $CD56^+$ $CD16^+$ and $CD56^+$ $CD16^-$ was 0.710 ($p = 0.001$, 95% CI: 0.596–0.824) and 0.658 ($p = 0.015$, 95% CI: 0.537–0.779), respectively. Optimal cut-off value determined from the ROC analysis for $CD56^+CD16^+$ and $CD56^+$ $CD16^-$ NK cells was 5.25%. and ≥3.4 respectively. The positive predictive value of having >1.6.% $CD56^+CD16^+$ and >1.01.% $CD56^+$ $CD16^-$ NK cells was 62% and 59%, and the negative predictive value of having 0.4% $CD56^+CD16^+$ and 0.55% CD56 + CD16- NK cells or less was 71% and 65% respectively.

Table 1
Comparison of the cytokines levels in recurrent miscarriage and fertile groups.

Cytokines[b]	Recurrentmisc arriage (n = 40)	Fertile (n = 40)	*p*[a]
IL[c]-2	11.63 ± 17.28	10.16 ± 15.23	0.930
IL-12	0.60 ± 0.97	0.48 ± 0.65	0.788

[a] The data were assessed using t-tests and Mann–Whitney U test.
[b] The values are mean ± S.D.
[c] IL = Interleukin.

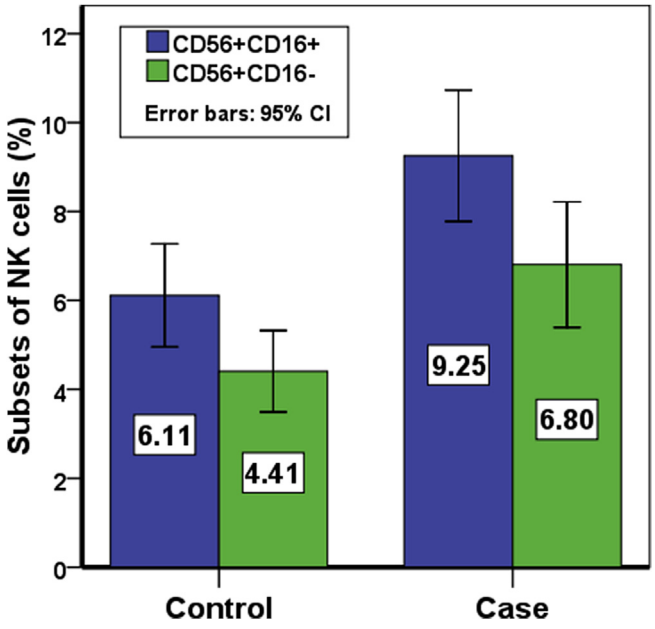

Fig. 2. Comparison of peripheral blood NK* cell levels in case** and control*** groups. *Natural killer cells. **Recurrent miscarriage. ***Fertile.

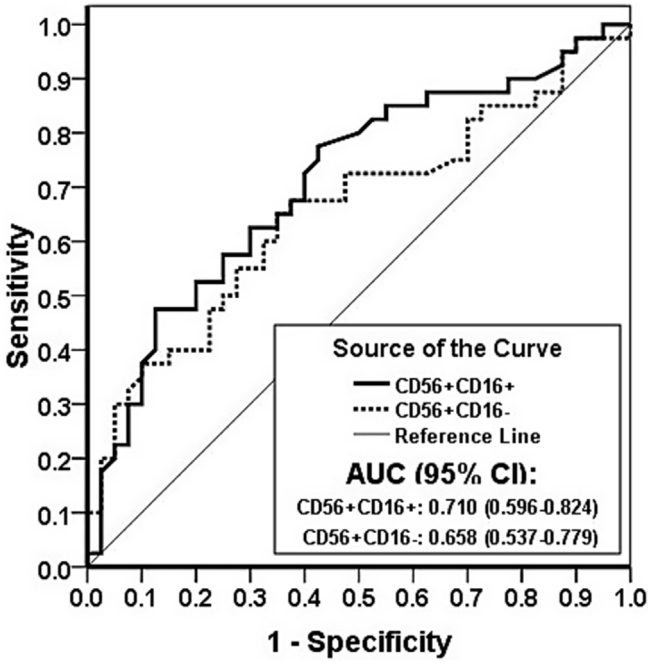

Fig. 3. ROC curve for $CD56^+$ $CD16^+$ and $CD56^+$ $CD16^-$ percentages in the prediction of unexplained recurrent miscarriage.

4. Discussion

Studies have shown a strong link between the immune system and miscarriage.[16] Abnormal cellular immunity, such as unusual levels of the NK cells, Th1, and cytotoxicity of the NK cells are recurrent abortion factors.[19] On the other hand, the relationship between peripheral blood NK cells and reproductive failure is one of the most arguably fields in reproductive health.[20] In this study, we found that there is a significant difference in $CD56^+$ $CD16^+$ and $CD56^+$ $CD16^-$ between the case and control groups, and level of NK cell subsets was higher in women with recurrent miscarriage.

Our study supports previous researches that women with RM have increased peripheral blood NK cells compared with control women. King et al. reported that NK cell percentage in women with RM was significantly higher than controls.[19] In another study, results showed that percentages of PB $CD16^-$, $CD45RO^-$, and $CD56^+CD16^+CCR7^+$ subsets were significantly higher in recurrent abortion members.[21] Furthermore, the findings in one study showed that frequencies of CD56 cells and NK cytotoxicity in PB for recurrent spontaneous abortion or *in vitro* fertilization failure were significantly higher than control groups.[22]

On the results from our study, we should mention which immune system suppression is needed for successful pregnancy, definitely, Cochrane review and other studies showed that immunoglobulin treatment does not increase live birth rate in women with recurrent miscarriage.[23,24] In contrast to our study, a number of studies have shown that many women with recurrent abortion do not have high NK cell levels.[25,26] This means that NK cell detection would be an inoperative method to recognize women with recurrent abortion from the common population, but is able to efficiently recognize a subpopulation of women with recognized recurrent abortion who may gain from immunosuppressive treatment.

In our study, a cut-off value of $\geq$5.25% ($p < 0.001$) and $\geq$3.4% ($p < 0.015$) for the increased percentage of $CD56^+$ $CD16^+$ and $CD56^+$ $CD16^-$cells in the PB becomes predictive of recurrent miscarriage. In Sugiura-Ogasawara et al.'s study, cut-off value of 3.75% of the increased percentage of $CD3^+$ $CD56^+$ $CD16^+$ NKT-like cells becomes predictive of recurrent miscarriage.

In the present study, there was no significant difference in the IL-2 and IL-12 between the two groups. In one study, results revealed that IL-2 in the patients with recurrent abortion was significantly higher than control group.[27] In another study, IL-2 in women with recurrent miscarriage and infertility was higher than control group, and immunization with paternal lymphocytes (PLI) was not associated with shift towards Th2 and the success of the next pregnancy.[28] Besides, Comba et al. reported that IL-12 in the blood and endometrial biopsy in women with recurrent abortion was significantly higher than the control group.[6]

It should be noted that moderate inflammation reaction can result in the maintenance of pregnancy, but abortion is due to severe form of the inflammation.[29] In most women with normal pregnancies, Th2 response overcomes the trophoblastic unknown antigens. In a large number of women with recurrent miscarriage, inflammatory response in the Th1 may be problematic for embryo developments.[30] However, in our study, the interleukin levels did not differ significantly between the two groups. It should be noted that this can be due to sampling time (from three months to one year after abortion or childbirth).

One of the strengths of our study was the concurrent evaluation of NK cells and cytokines (Th1) in the two groups that have been mentioned in immunological studies and topics as recurrent abortion factor. Therefore, we consider that the increased NK cells and no cytokines (Th1) in the PB may result in recurrent miscarriage. The limitation of our study was that the case sample only included women who had experienced recurrent miscarriage in the first trimester due to the absence of cases of abortion in the second trimester. Therefore, it is suggested that future studies include the immunologic assessment of women who experience a miscarriage in the second trimester.

In conclusion, we found increased NK cells in the PB of women with recurrent miscarriage. The relationship of miscarriage with an increased percentage of $CD56^+$ $CD16^+$ and $CD56^+$ $CD16^-$ cells in PB strongly establishes prospective researches to recognize the predictive value of these parameters in evaluation patients with recurrent miscarriage.

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