Endometrial CD16$^+$ and CD16$^-$ NK Cell Count in Fertility and Unexplained Infertility

Gisela Junovich[1], Agustina Azpiroz[1], Eugenia Incera[1], Constanza Ferrer[2], Agustin Pasqualini[1], Gabriela Gutierrez[1,2]

[1]Halitus Medical Institute, Buenos Aires, Argentina;
[2]Fundación Repro, Buenos Aires, Argentina

Keywords
IL-6, Implantation failure, NK cells, unexplained infertility, VEGF

Correspondence
Gabriela Gutierrez, Halitus Medical Institute, Buenos Aires (1122), Argentina.
E-mail: gabriela.gutierrez@halitus.com

Submission November 12, 2012; accepted April 3, 2013.

Citation
Junovich G, Azpiroz A, Incera E, Ferrer C, Pasqualini A, Gutierrez G. Endometrial CD16$^+$ and CD16$^-$ NK cell count in fertility and unexplained infertility. Am J Reprod Immunol 2013

doi:10.1111/aji.12132

Problem
Peripheral counts of CD16$^+$ NK cells have been well characterized in reproductive failure. However, not enough case-control clinical studies have been conducted to establish normal or abnormal CD16$^{+/-}$ values in the endometrium.

Method of study
Peripheral and endometrial NK cell counts by FACS, IL-6, and VEGF cytokines levels by ELISA were characterized in fertile women and unexplained infertility patients with implantation failures (UI-IF) during implantation window. ROC and correlation analysis were performed.

Results
Receiver Operating Characteristic(ROC) analysis revealed endometrial CD16$^+$ NK cells, IL-6, and VEGF as good diagnostic parameters for unexplained infertility. Almost half of UI-FI patients showed increased total and CD16$^+$ NK cell counts correlating with decreased levels of endometrial IL-6 and VEGF. No correlation was found with peripheral blood values.

Conclusion
Increased CD16$^+$ NK cells were associated with IL-6 and VEGF deficiency in a high proportion of UI-IF patients. Testing for these immunomarkers could be a potential tool in infertility diagnosis.

Introduction

A correct etiological diagnosis of infertility is essential to improve the outcome of assisted reproduction treatments, taking into account that in up to 50% of cases, the cause of infertility cannot be identified.[1,2] As several studies suggest that immunological testing is necessary for the management of infertility,[3] natural killer (NK) cells are being included as they constitute the most abundant leukocyte population in the decidua.[4] These cells can be classified into two subsets that differ in their cell surface markers as well as in functional properties: the cytotoxic CD56^{dim} CD16$^+$ subset and the angiogenic CD56^{bright} CD16$^-$.[5–7] While 90% of peripheral blood NK cells belong to the cytotoxic subset,[6] CD16$^-$ NK cells predominate in the endometrium during the secretory menstrual phase. Migration of NK cells to the uterus as well as endometrial differentiation from CD34$^+$ stem cell precursors has been postulated as of CD16$^-$ origin.[8–10] Moreover, studies have demonstrated that CD9 is a useful marker to discriminate endometrial from peripheral blood NK cells.[11,12] It has also been suggested that CD16$^-$ CD9$^+$ NK cells

have an important role in the development and remodeling of uterine spiral arteries, an essential process for normal pregnancy.[13] Although NK cell counts in peripheral blood of women with infertility have become a common practice in many centers of reproductive medicine, there are still few clinical studies that evaluate the correlation of endometrial with peripheral NK cell counts or establish normal values. Additionally, studies performed in implantation failure patients are scarce, whereas most studies have been undertaken in women who have undergone recurrent spontaneous abortions.[14–16]

Considering that peripheral blood NK cell numbers do not necessarily correlate with increased NK activity,[7] further investigation is needed to determine endometrial activity in infertility. A proper balance between inflammatory/anti-inflammatory cytokine expression[17,18] and the association with $CD16^+$/$CD16^-$ NK cell counts could be a potential tool for immunological diagnosis of infertility.

Interleukin-6 (IL-6) plays a crucial role in implantation, and deficient levels have been associated with pregnancy losses.[19–21] Moreover, the presence of this regulatory cytokine leads the Th1/Th2 balance toward Th2 cytokine production.[22] Up to date, there is no evidence associating IL-6 levels with endometrial NK cell counts.

Taking into account that the endometrium is a source of angiogenic factors and that $CD16^-$ NK cells express protein and RNA messenger of the vascular endothelial growth factor C (VEGF-C), it has been suggested that this NK cell subset could be involved in endometrial angiogenesis.[23] VEGF is a specific mitogen that promotes angiogenesis of the placental vasculature, as well as maternal and embryonic endothelial cell proliferation.[24] Even though the deficiency of this factor has been associated with recurrent abortion.[25] its levels have not been associated with endometrial NK cell $CD16^-$ counts in fertile and infertile patients.

The aim of our study was to determine uterine and systemic values of $CD16^{+/-}$ NK cells, IL-6, and VEGF during the implantation window, providing new biomarkers for the diagnosis of immunological factors in unexplained infertility. It was also designed to characterize and to establish a correlation between the number and phenotype of endometrial versus peripheral blood NK cells in unexplained infertility patients with repetitive implantation failure (UI-IF) as compared to data obtained from fertile women.

Materials and methods

Study Subjects

This study was performed according to the Declaration of Helsinki for Medical Research, with appropriate ethical approval by Ethics Committee of Cer Institute (CECIC), Buenos Aires, Argentina.

From August 2008 to August 2010, 44 patients were recruited after providing written informed consent.

The study group comprised 26 IVF patients with unexplained infertility who had failed ≥ 2 IVF-ICSI treatments with ≥ 3 good-quality embryos transferred (UI-IF). The control group comprised 18 oocyte donor fertile women.

Inclusion criteria for the study group were as follows: 21–38 years old, primary infertility (no live birth) or secondary infertility (live birth at least 2 years before), regular menstrual cycle (24–35 days), body mass index (BMI) ≤ 26, FSH <10 mUI/mL, E2 < 50 pg/ml on day 2–3 of the menstrual cycle, normal karyotype, previous stimulation cycles with mature oocytes (MII) ≥ 5, and ≥ 3 good-quality embryos transferred in ≥ 2 cycles of assisted reproductive techniques.

Inclusion criteria for control group were as follows: 21–35 years old, ≥ 1 live birth younger than 2 years old, regular menstrual cycle (24–35 days), BMI ≤ 26, FSH <10 mUI/mL, E2 < 50 pg/mL on day 2–3 of the menstrual cycle, normal karyotype, previous stimulation cycles with mature oocytes (MII) ≥ 12.

Women with endometritis, endometriosis, tubal factor, ovulatory dysfunction, anatomical uterine pathologies, and abnormal male factor (according to the reference values for healthy human semen established in the 1999 World Health Organization guidelines) were excluded.

The differences in the number of patients tested for cytokines or NK cells depend on number of cells obtained from the biopsy. Most of the times, sample is not enough to determine all parameters.

Endometrial Samples

Endometrial biopsies and peripheral blood samples were obtained 5–9 days after ovulation determined by ultrasound scan (implantation window) from all included women in a non-conception cycle without any hormonal treatment. No hormonal methods of

contraception were used during this cycle. Any hormonal treatment was avoided during at least one previous cycle from the last failed IVF cycle to the time of testing.

Endometrial tissue samples were obtained by performing a biopsy using Pipelle Cornier's catheters. Samples were collected into 4 mL of RPMI 1640 medium (Gibco, Carlsbad, CA, USA) containing 10% Fetal Calf Serum (FCS, GIBCO) and penicillin/streptomycin (100 IU/mL and 100 μg/mL, respectively, GIBCO).

Peripheral Leukocyte Separation

Blood samples were taken simultaneously with the endometrial biopsy. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Histopaque-1077 density gradient according to manufacturer's directions (Sigma-Aldrich, St Louis, MO, USA).

Endometrial Cell Suspension

Endometrial cells were prepared for subsequent analysis. Briefly, samples were disrupted mechanically. Cell suspension was strained by a 100 μm mesh and incubated for 10 min at 37°C with 1 mL NH_4Cl to lyse red blood cells.

Flow Cytometry

The following monoclonal antibodies (mAbs) conjugated with FITC, PE, or PECy5 were used to analyze the phenotype of PBMC and endometrial cells. For the PBMC analysis, specific commercial antibodies (BD, NJ, USA) anti-CD3 (FITC), anti-CD16 (PECy5), and anti-CD56 (PE) were used. For endometrial NK cells analysis, specific commercial antibodies (BD, NJ, USA) anti-CD9 (FITC), anti-CD16 (PECy5), and anti-CD56 (PE) were used. $CD56^+CD9^+$ expression allows to differentiate endometrial NK cells from peripheral $CD56^+CD9^-$ NK cells.[11,12] A total of 1×10^6 cells were incubated with 10 μL of anti-CD16, anti-CD3, or anti-CD9 and 15 μL of anti-CD56 for 30 min at room temperature in the dark. Cells were then washed with phosphate-buffered saline (PBS) and fixed with 0.02% formaldehyde (Carlo Erba, Italy) in PBS. Immunofluorescence reactivity was analyzed in a FACS ARIA Cells Sorter Cytometer (BD, USA). For isotype controls, the corresponding murine immunoglobulin conjugated with FITC, PE, and PECy5 (BD, NJ, USA) was used.

Analysis of Peripheral NK Cell Subsets

A tight gate in the lymphocyte region of FSC versus SSC graph was drawn. The number of $CD56^+CD3^-$ cells was calculated as% of total lymphocytes. Numbers of cells were determined by% of $CD56^+CD3^-$ cells. The ratio of $CD56^+CD16^{+/-}$subsets was reported as a percentage of over total lymphocytes.

Analysis of Endometrial NK Cell Subsets

To establish the most representative FACS analysis, we analyzed statistical distribution of percentage of $CD56^+CD9^+$ from 0.2 to 2 x 10^5 total endometrial cells. Best reproducible result was obtained from 1×10^5 cells (data not shown). The ratio of $CD56^+CD16^{+/-}$ subsets was reported as a percentage over 1×10^5 endometrial cells.

Cell Culture Supernatant

Endometrial and peripheral blood cells (4×10^5) were cultured in 96-well tissue culture plates in 150 μL RPMI medium, supplemented with 10% FCS and 1% PS. Cells were incubated at 37°C and 5% CO_2 humid atmosphere during 48 hr. Supernatants were collected and centrifuged at 2000 × ***g*** during 10 min and then at 8000 × ***g*** during 10 min. They were stored at −70°C until cytokine analysis was performed.

IL-6 and VEGF Quantification by ELISA

Levels of total soluble IL-6 and VEGF produced by endometrial and PMBC cells were determined by a double antibody sandwich ELISA using commercial kits (R&D, Minneapolis, MN, USA). Briefly, 96-well plates were coated with anti-IL-6 or anti-VEGF monoclonal antibodies and kept overnight at room temperature (RT). After washing with PBS, free sites were blocked with 1% bovine serum albumin (BSA, SIGMA, St Louis, MO, USA) in PBS and incubated at RT for 1 hr. Washes were repeated with PBS supplemented with 0.05% Tween-20 (Anedra, Buenos Aires, Argentina). Dilutions of 1/20-1/50 IL-6 samples and undiluted samples of VEGF were incubated for 2 hr at RT. Biotinylated detection antibody was incubated at RT for 1 hr after washing. Washes were repeated and streptavidin-HRP solution was incubated for 20 min at RT. Color reaction was

developed by the addition of 3,3′,5,5′-tetramethylbenzidine (TMB, MP Biomedicals, OH, USA) and stopped with 1M H_2SO_4 solution. Optical densities were read at 450–620 nm using an automatic plate reader (Metertech, Austria). Levels of IL-6 (pg/ml) and VEGF (pg/ml) were calculated by linear regression using a standard curve constructed with human recombinant IL-6 and VEGF provided by the manufacturer.

Statistical Analysis

Considering our published previous data,[26] sample size for UI-FI and fertile woman group was determined using Epidat 3.1 software (Collaboration agreement signed by the Panamerican Health Organization and the Ministry of health of the Xunta of Galicia).

After testing for Gaussian distribution and variance equality, means were compared by Mann–Whitney's *U*-test for nonparametric distributions and Student's *t*-test for variables displaying a normal distribution, using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Data were expressed as mean ± S.D. Differences were considered significant when $P < 0.05$. When difference was significant, the sample size required for an 80% potency was calculated, and it was expressed as Sample Size representative.

Diagnostic accuracy of evaluated variables was assessed by calculating the area under the ROC (Receiver Operating Characteristic) curve. ROC curve represents the probability of true positive results (sensitivity) as a function of the probability of false positive results (1- specificity). The area under the ROC curves was compared employing the MedCalc software (version 11.3.0; Ostend, Belgium). Parameters with area under the curve (AUC) >0.6 and $P < 0.05$ were considered as good discriminators parameters.

The Pearson's (Gaussian) or Spearman's (non-Gaussian) tests of correlation were performed employing MedCalc. Correlation coefficients (r) were considered significant when $P < 0.05$.

Results

Endometrial NK Cell Counts in Fertile and Infertile Women

Patients with UI-IF showed increased percentage of endometrial NK cells as well as $CD16^+$ absolute count when compared with fertile women (*$P = 0.022$ Fig. 1a and ** $P = 0.009$ Fig. 1b, respectively. Sample size representative = 25). No significant difference in $CD16^-$ NK cell absolute count was observed between groups (5.1 ± 6.1 versus 2.0 ± 1.5 data not shown. Sample size representative = 31). Moreover, a negative linear correlation was found between endometrial% $CD56^+CD9^+$ and peripheral% $CD56^+CD3^-$ NK cells considering total population (UI-IF + fertile) ($r = -0.3197$ * $P = 0.04$; Fig. 1c), whereas no correlation was found between endometrial and peripheral $CD16^+$ NK cells considering total population ($r = -0.2927$ $P > 0.05$; Fig. 1d).

IL-6 and VEGF Production by PBMC and Endometrial Cells in Fertile and Infertile Women

Patients with UI-IF showed a significant deficiency in IL-6 levels in endometrial (**$P = 0.001$ Fig. 2a. Sample size representative = 22) and PBMC culture cell supernatant (**$P = 0.0027$ graph not shown. Sample size representative = 16) with regard to fertile women. A positive linear correlation between uterine and peripheral IL-6 levels was found considering UI-IF and fertile women ($n = 29$ $r = 0.4010$ * $P = 0.03$; Fig. 2b). Moreover, a significant negative correlation between endometrial $CD16^+$ NK cell absolute count and endometrial IL-6 protein expression was found considering UI-IF and fertile women ($n = 28$ $r = -0.4051$ *$P = 0.03$; Fig. 2c).

Patients with UI-IF also displayed a significant deficiency in endometrial VEGF level compared with fertile women (**$P = 0.0062$ Fig. 3a. Sample size representative = 13) with a positive significant linear correlation with peripheral levels considering UI-IF and fertile women ($n = 28$ $r = 0.5122$ ** $P = 0.005$; Fig. 3b) and a negative significant correlation with endometrial $CD16^+$ NK cell absolute count considering UI-IF and fertile women ($n = 31$ $r = -.4153$ *$P = 0.0225$; Fig. 3c).

Diagnostic Test of Endometrial Parameters

The AUC values for% $CD56^+CD9^+$ (AUC = 0.705 **$P = 0.0096$), absolute count of $CD16^+$ NK cells (AUC = 0.735 **$P = 0.002$), endometrial protein levels of IL-6 (AUC = 0.848 **$P = 0.0001$), and VEGF (AUC = 0.817 **$P = 0.0001$) showed a good capacity to discriminate between a fertile and infertile endometrium (Fig. 4a–d).

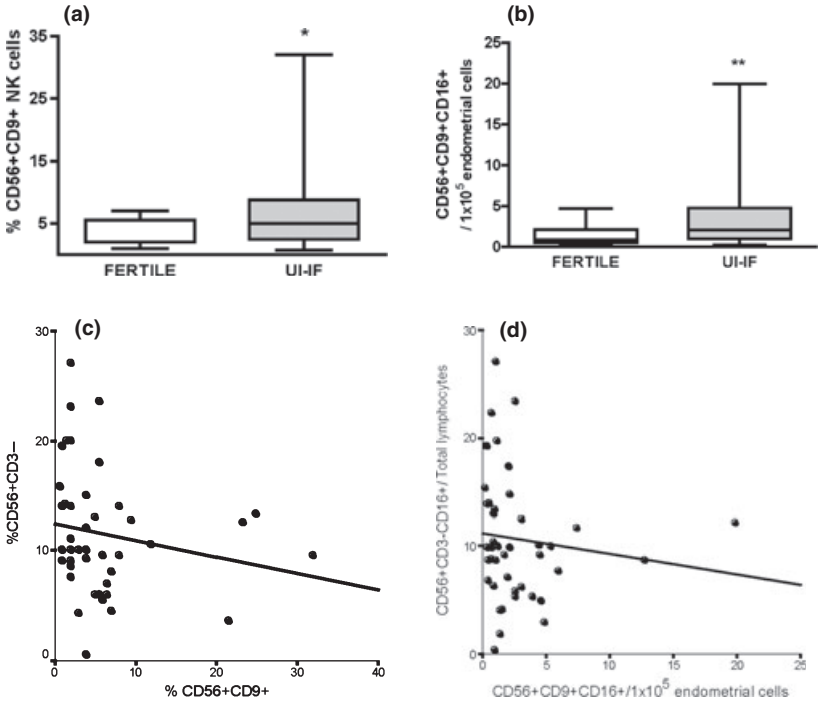

Fig. 1 Endometrial NK cell counts in fertile and infertile women. (a) Percentage of total endometrial NK cells (% CD56$^+$CD9$^+$) from UI-IF patients (n = 26) versus fertile women (n = 18) (*P = 0.02). Median is overlapped with percentile 25 in fertile women group. (b) Absolute count of CD16$^+$ NK cells (CD56$^+$CD9$^+$CD16$^+$/1 × 10^5 endometrial cells) from UI-IF patients (n = 26) versus fertile women (n = 18) (**P = 0.009). Median is overlapped with percentile 25 in fertile women group. (c) Correlation (r = −0.3197 P = 0.04) between endometrial (% CD56$^+$CD9$^+$) and peripheral blood (% CD56$^+$CD3$^-$) NK cells. (d) Correlation (r = −0.2927 P > 0.05) between endometrial (CD56$^+$CD9$^+$CD16$^+$/1 × 10^5 endometrial cells) and peripheral blood (CD56$^+$CD3$^-$CD16$^+$/total lymphocytes) absolute NK cell count.

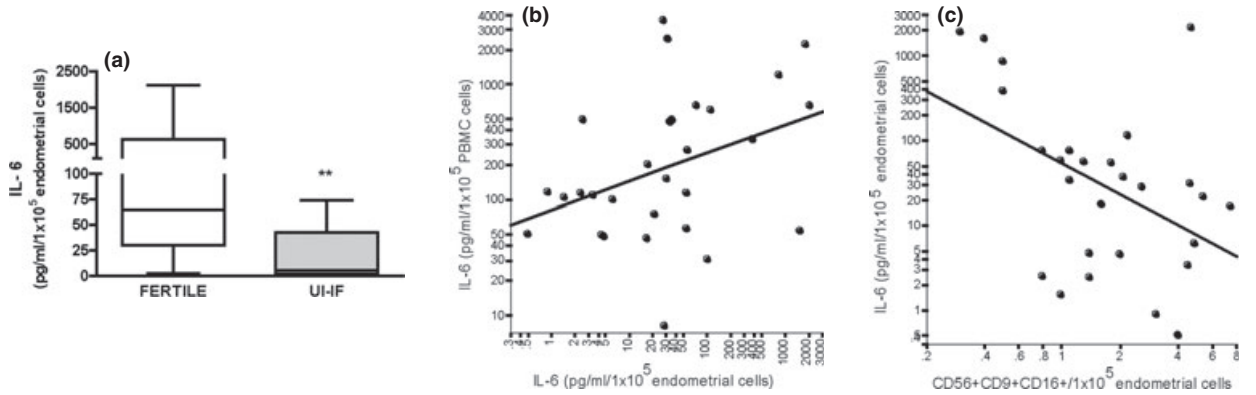

Fig. 2 IL-6 production by PBMC and endometrial cells in fertile and infertile women. (a) IL-6 protein levels in endometrial cell culture supernatant from UI-IF patients (n = 14) versus fertile women (n = 16) (**P = 0.001). (b) Correlation (r = 0.4010 P = 0.03) between IL-6 levels in culture supernatant of endometrial and PBMC. (c) Correlation (r = −0.4051 P = 0.03) between protein levels of IL-6 in endometrial cells culture supernatant and absolute count of endometrial CD16$^+$ NK cell (CD56$^+$CD9$^+$CD16$^+$/1 × 10^5 endometrial cells).

Discussion

The lack of an accurate diagnosis in reproductive medicine leads to treatment failure. Among potential biochemical diagnostic markers, cytokines and immune cells constitute important tools to differentiate treatment groups. Moreover, natural killer (NK) cell count is one of the best characterized immunological marker in reproductive failure. For this reason, new treatments are designed to modulate the number and function of these cells in infertile patients. However, more studies need to be conducted to characterize and differentiate endometrial NK cells in different reproductive pathologies such as recurrent spontaneous abortion and implantation failure. Most published data based on abnormal

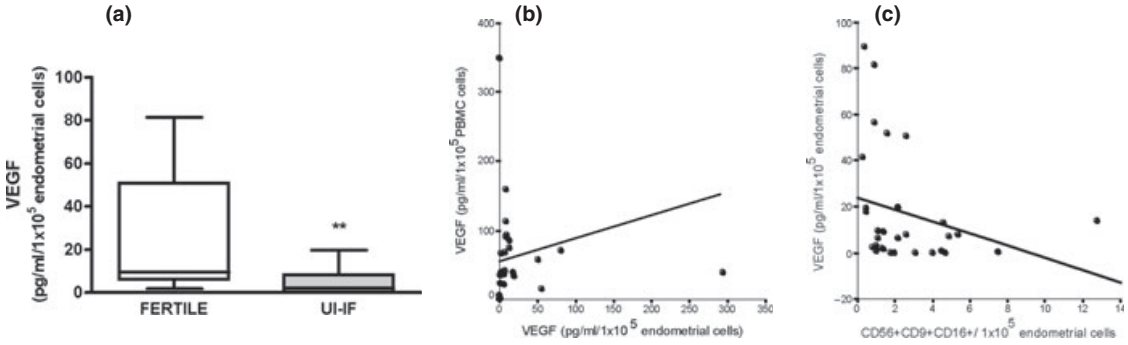

Fig. 3 VEGF production by PBMC and endometrial cells in fertile and infertile women. (a) VEGF protein levels in endometrial cell culture supernatant from UI-IF patients (n = 16) versus fertile women (n = 13) (**P = 0.0062). (b) Correlation (r = 0.5122 P = 0.005) between levels of VEGF in culture supernatant of endometrial and PBMC cells. (c) Correlation (r = −0.4153 P = 0.0225) between protein levels of VEGF in endometrial cell culture supernatant and absolute count of endometrial $CD16^+$ NK cells ($CD56^+CD9^+CD16^+/1 \times 10^5$ endometrial cells).

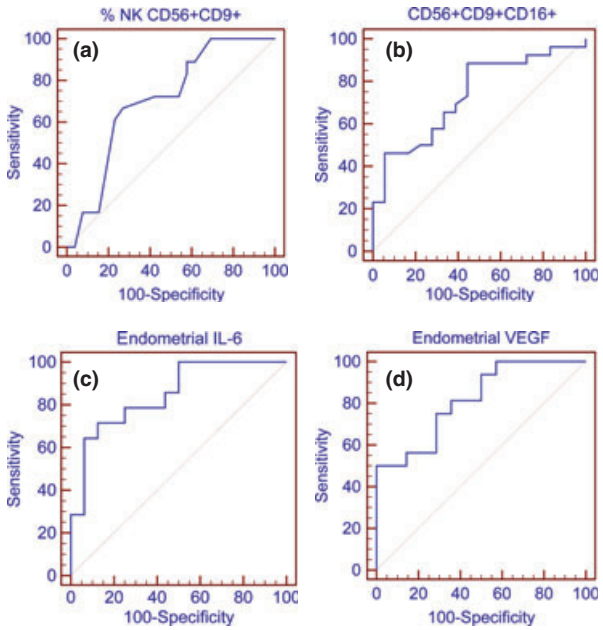

Fig. 4 Receiver Operating Characteristic (ROC) curves analysis of biochemical endometrial markers of unexplained infertility. (a) ROC curve of% CD56 + CD9 + cells ($n_{UI\text{-}IF+fertil}$ = 44 AUC = 0.705 P = **0.0096; sensitivity = 46.2; 1-specificity = 27.8). (b) ROC curve of $CD56^+CD9^+CD16^+$ NK cells ($n_{UI\text{-}IF+fertil}$ = 44 AUC = 0.735 **P = 0.002 sensitivity = 46.2; 1-specificity = 5.5). (c) ROC curve of endometrial IL-6 levels ($n_{UI\text{-}IF+fertil}$ = 30 AUC = 0.848 **P = 0.0001 sensitivity = 64.3; 1-specificity = 6.2). (d) ROC curve of endometrial VEGF levels ($n_{UI\text{-}IF+fertil}$ = 29 AUC=0.817 **P = 0.0001 sensitivity = 56.2; 1-specificity = 14.3).

counts of NK cell were performed in peripheral blood of patients with recurrent abortions.[27] These studies were based on the speculation that the imbalance of peripheral blood NK cell reflects an alteration in the endometrium.[4] Moreover, there is not enough evidence regarding normal values of endometrial $CD16^+$ and $CD16^-$ NK cells, NK subpopulations, and NK cell markers. Most studies on endometrial NK cells have been performed by histological evaluation, where differences in NK cell subsets are limited while studies using flow cytometry provide a much better analysis of NK cell populations.[7] Furthermore, functional parameters should be tested to associate NK cell counts with NK cell activity. In this sense, we have previously postulated that the switch from an inflammatory to an anti-inflammatory response may modulate the ratio between $CD16^+/CD16^-$ NK cell count.[26] It has been published that there is a delicate balance and a shift from an inflammatory to an anti-inflammatory response during the transition from implantation to placental vascularization,[17,18] and this could be exerted by IL-6.[22,26] This regulatory cytokine also plays a crucial role in implantation,[19] and it has been published that IL-6 stimulates VEGF production.[28–30] On the other hand, VEGF is implicated in endometrial angiogenesis, and it is produced by $CD16^-$ NK cells.[23,31]

Therefore, the present study was focused on endometrial NK cell characterization as a possible diagnostic factor of unexplained infertility with implantation failures (UI-IF). We compared endometrial and peripheral blood values in fertile women and UI-IF patients. Finally, we investigated uterine and peripheral expression of IL-6 and VEGF in primary cells ex vivo as potential markers of regulatory and angiogenic response.

After determining normal values in fertile women, we found that almost half of the studied population showed exacerbated counts of cytotoxic CD16$^+$ endometrial NK cells. No correlation between peripheral and uterine CD16$^+$ NK cell counts was found either for fertile or infertile patients. Therefore, peripheral NK cell testing in UI-IF patients should be revalidated. The differences observed in NK cell counts could be attributed to their playing a different role from that of endometrial NK cells under the potential influence of other factors such as stress or infections.[4]

Herein, we determined normal endometrial IL-6 and VEGF values and both of them correlate with their counterpart in peripheral blood. Half of UI-IF patients express deficient levels of IL-6 and VEGF in peripheral blood and endometrial culture supernatant. Moreover, we observed that increased levels of endometrial CD16$^+$ NK cell were associated with peripheral and uterine deficiency of IL-6 and VEGF.

Considering these data, we postulate that deficient IL-6 expression may lead to an increase in cytotoxic response mediated by high endometrial CD16$^+$ NK cell count. Additionally, implantation failure in these patients could be related to an inadequate endometrial vascularization as a consequence of a deficient VEGF expression. These data are also consistent with previous results demonstrating an association between IL-6 and VEGF deficiency in animal fetal loss.[32,33]

Further analysis with larger sample size should be conducted to test this hypothesis and to determine the clinical importance of endometrial NK cells, IL-6, and VEGF testing in improving pregnancy and take-home baby rates; however, we are able to postulate IL-6, VEGF, and uterine–not peripheral–NK cell subsets, as good diagnostic markers of unexplained infertility.

References

1 Assisted Reproductive Technology and Intrauterine Inseminations in Europe 2006: Results generated from European registers by ESHRE. *Hum Reprod* 2009; 1:1–21.

2 Matthiesen L, Kalkunte S, Sharma S: Multiple pregnancy failures: an immunological paradigm. *Am J Reprod Immunol* 2012; 67:334–340.

3 Kwak Kim J, Yang K, Gilman-Sachs A: Recurrent pregnancy loss: a disease of inflammation and coagulation. *J Obstet Gynecol Res* 2009; 35:609–622.

4 Moffett-King A: Natural killer cells and pregnancy. *Nat Rev Immunol* 2002; 2:656–663. Review. Erratum in: Nat Rev Immunol 2002; 2:975.

5 Starkey P, Sargent I, Redman C: Cell populations in human early pregnancy decidua: characterization and isolation of large granular lymphocytes by flow cytometry. *Immunology* 1988; 65: 129–134.

6 Cooper M, Fehniger T, Caligiuri M: The biology of human natural killer-cell subsets. *Trends Immunol* 2001; 22:633–640. Review.

7 Kwak-Kim J, Gilman-Sachs A: Clinical implication of natural killer cells and reproduction. *Am J Reprod Immunol* 2008; 59:388–400. Review.

8 Dosiou C, Giudice L: Natural killer cells in pregnancy and recurrent pregnancy loss: endocrine and immunologic perspectives. *Endocr Rev* 2005; 26:44–62. Review.

9 Carlino C, Stabile H, Morrone S, Bulla R, Soriani A, Agostinis C, Bossi F, Mocci C, Sarazani F, Tedesco F, Santoni A, Gismondi A: Recruitment of circulating NK cells through decidual tissues: a possible mechanism controlling NK cell accumulation in the uterus during early pregnancy. *Blood* 2008; 111:3108–3115.

10 Vacca P, Vitale CH, Montaldo E, Conte R, Cantoni C, Fulcheri E, Darreta V, Moretta L, Mingari C: CD34 + hematopoietic precursors are present in human decidua and differentiate into natural killer cells upon interaction with stromal cells. *PNAS* 2011; 108:2402–2407.

11 Koopman L, Kopcow H, Rybalov B, Boyson J, Orange J, Schatz F, Masch R, Lockwood C, Schachter A, Park P, Strominger J: Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J Exp Med* 2003; 198:1201–1212.

12 Mselle T, Meadows S, Eriksson M, Smith J, Shen L, Wira C, Sentman C: Unique characteristics of NK cells throughout the human female reproductive tract. *Clin Immunol* 2007; 124:69–76.

13 Bulmer J, Lash G: Human uterine natural killer cells: a reappraisal. *Mol Immunol* 2005; 42:511–521. Review.

14 Coulam C, Goodman C, Roussev R, Thomason E, Beaman K: Systemic CD56 + cells can predict pregnancy outcome. *Am J Reprod Immunol* 1995; 33:40.

15 Ntrivalas E, Kwak-Kim J, Gilman-Sachs A, Chung-Bang H, Ng S, Beaman K, Mantouvalos HP, Beer A: Status of peripheral blood natural killer cells in women with recurrent spontaneous abortions and infertility of unknown aetiology. *Hum Reprod* 2001; 16:855–861.

16 Park D, Lee H, Park C, Hong S, Kwak-Kim J, Yang K: Peripheral blood NK cells reflect changes in decidual NK cells in women with recurrent miscarriages. *Am J Reprod Immunol* 2010; 63:173–180.

17 Chaouat G, Zourbas S, Ostojic S, Lappree-Delage G, Dubanchet S, Ledee N, Martal J: A brief review of recent data on some cytokine expressions at materno-fetal interface which might challenge the classical Th1/Th2 dichotomy. *J Reprod Immunol* 2002; 53:241–256.

18 Guzeloglu-Kayisli O, Kayisli U, Taylor H: The role of growth factors and cytokines during implantation: endocrine and paracrine interactions. *Semin Reprod Med* 2009; 27:62–79. Review.

19 Tabibzadeh S, Kong Q, Babaknia A, May L: Progressive rise in the expression of interleukin-6 in human endometrium during menstrual cycle is initiated during the implantation window. *Mol Hum Reprod* 1995; 1:2793–2799.

20 Robertson S, O'Connell A, Ramsay A: The effect of IL-6 deficiency on implantation, fetal development and parturition in mice. Proc of the 31st Annual Conference of the Australian Society for Reproductive Biology, Canberra, Australia, 2000, p 97 (Abstract 97).

21 Dimitriadis E, White C, Jones R, Salamonsen L: Cytokines, chemokines and growth factors in endometrium related to implantation. *Hum Reprod Update* 2005; 11:613–630. Review.

22 Diehl S, Rincón M: The two faces of IL-6 on Th1/Th2 differentiation. *Molecular Immunol* 2002; 39:531–536.
23 Li X, Charnock-Jones D, Zhang E, Hiby S, Malik S, Day K, Licence D, Bowen J, Gardner L, King A, Loke Y, Smith S: Angiogenic growth factor messenger ribonucleic acids in uterine natural killer cells. *J Clin Endocrinol Metab* 2001; 86:1823–1834.
24 Galazios G, Papazoglou D, Tsikouras P, Kolios G: Vascular endothelial growth factor gene polymorphisms and pregnancy. *J Matern Fetal Neonatal Med* 2009; 22:371–378.
25 Vourela P, Carpen O, Tulppala M: Halmesmaki: VEGF, its receptor and the tie receptors in recurrent miscarriage. *Mol Hum Reprod* 2000; 6:276–282.
26 Junovich G, Mayer Y, Azpiroz A, Daher S, Iglesias A, Zylverstein C, Gentile T, Pasqualini S, Markert U, Gutiérrez G: Ovarian stimulation affects the levels of regulatory endometrial NK cells and angiogenic cytokine VEGF. *Am J Reprod Immunol* 2011; 65: 146–153.
27 Tang A, Alfirevic Z, Quenby S: Natural killer cells and pregnancy outcomes in women with recurrent miscarriage and infertility: a systematic review. *Hum Reprod* 2011; 26:1971–1980. Review.
28 Cohen T, Nahari D, Cerem LW, Neufeld G, Levi BZ: Interleukin 6 induces the expression of vascular endothelial growth factor. *J Biol Chem* 1996; 271:736–741.
29 Wei L, Kuo M, Chen C, Chou C, Lai KB, Lee C, Hsieh C: Interleukin−6 promotes cervical tumor growth by VEGF–dependent angiogenesis via a STAT3 pathway. *Oncogene* 2003; 22:1517–1527.
30 Sen A, Paine SK, Chowdhury IH, Mukherjee A, Choudhuri S, Saha A, Mandal LK, Bhattacharya B: Impact of interleukin-6 promoter polymorphism and serum interleukin-6 level on the acute inflammation and neovascularization stages of patients with Eales' disease. *Mol Vis* 2011; 17:2552–2563.
31 Kalkunte S, Mselle T, Norris W, Wira C, Sentman C, Sharma S: Vascular endothelial growth factor C facilitates immune tolerance and endovascular activity of human uterine NK cells at the maternal-fetal interface. *J Immunol* 2009; 182:4085–4092.
32 Gutierrez G, Sarto A, Berod L, Canellada A, Gentile T, Pasqualini S, Margni R: Regulation of interleukin-6 fetoplacental levels could be involved in the protective effect of low-molecular weight heparin treatment on murine spontaneous abortion. *Am J Reprod Immunol* 2004; 51:160–165.
33 Junovich G, Dubinsky V, Gentile T, Sarto A, Pasqualini S, Gutiérrez G: Comparative immunological effect of anticoagulant and antioxidant therapy in the prevention of abortion in mice. *Am J Reprod Immunol* 2011; 65:104–109.