

# An increase in the absolute count of CD56<sup>dim</sup>CD16<sup>+</sup>CD69<sup>+</sup> NK cells in the peripheral blood is associated with a poorer IVF treatment and pregnancy outcome

M.Y.Thum<sup>1,2,3,4</sup>, S.Bhaskaran<sup>2</sup>, H.I.Abdalla<sup>1</sup>, B.Ford<sup>2</sup>, N.Sumar<sup>2</sup>, H.Shehata<sup>3</sup>  
and A.S.Bansal<sup>2</sup>

<sup>1</sup>Lister Fertility Clinic, Lister Hospital, Chelsea Bridge Road, London SW1W 8RH, <sup>2</sup>Immunology Department and <sup>3</sup>Women Health Department, Epsom and St Helier University Hospitals NHS Trust, Surrey, UK

<sup>4</sup>To whom correspondence should be addressed. E-mail: mythum@doctors.net.uk

**BACKGROUND:** Our aim was to evaluate the effect of the absolute count of the activation marker (CD69), IgG Fc receptor (CD16) and inhibitor marker (CD94) expression on peripheral blood natural killer (NK) cells on implantation and miscarriage rates after IVF treatment. **METHODS:** Prospective observational study of 138 randomly selected women who underwent IVF treatment from December 2002 to September 2003. NK cells were identified as CD56<sup>+</sup> (dim + bright) and CD3<sup>-</sup> by flow cytometry. The absolute counts of the CD69<sup>+</sup>, CD16<sup>+</sup> and CD94<sup>+</sup> expressing NK cells were recorded and their relation to IVF treatment outcome and miscarriage rate was analysed. **RESULTS:** The mean ( $\pm$ SD) absolute count of the CD56<sup>dim</sup>CD16<sup>+</sup>CD69<sup>+</sup> NK cells for women who had a successful ongoing pregnancy was  $0.61 \times 10^6/l$  ( $\pm 0.31$ ). For those women who failed to achieve a pregnancy, the mean value of the absolute count of CD56<sup>dim</sup>CD16<sup>+</sup>CD69<sup>+</sup> NK cells was significantly ( $P = 0.003$ ) higher at  $1.66 \times 10^6/l$  ( $\pm 0.52$ ). The absolute count of CD56<sup>dim</sup>CD16<sup>+</sup>CD94<sup>+</sup> and CD56<sup>dim</sup>CD16<sup>+</sup> NK cells did not show any statistically significant differences between those women with successful and failed IVF treatment. Receiver operating characteristic (ROC) curve analysis was performed to select a CD69 threshold for further statistical analysis. The implantation rate (IR) was significantly lower (13.1%) and miscarriage rate (MR) was significantly higher (66.7%) for women with an absolute CD56<sup>dim</sup>CD16<sup>+</sup>CD69<sup>+</sup> NK cell count of  $>1.0 \times 10^6/l$  compared to women with count below this value (IR 28.2% and MR 16.7%). Further analysis of the absolute count of CD56<sup>bright</sup>CD69<sup>+</sup> and CD56<sup>bright</sup>CD94<sup>+</sup> NK cells did not show any significant difference between those women with successful and failed IVF treatment. **CONCLUSIONS:** An increase in the absolute count of activated NK cells (CD56<sup>dim</sup>CD16<sup>+</sup>CD69<sup>+</sup>) in the peripheral blood is associated with a reduced rate of embryo implantation in IVF treatment. Furthermore, women with high CD56<sup>dim</sup>CD16<sup>+</sup>CD69<sup>+</sup> peripheral blood NK cell absolute count, who are able to achieve pregnancy, have a significantly higher miscarriage rate.

*Key words:* activation markers/CD69/flow cytometry/IVF/natural killer cells

## Introduction

The natural killer (NK) cell is the most abundant immune cell infiltrating the uterine implantation site (Moffett-King, 2002). It is the first line cellular immune defence mechanism and has a close contact with conceptus or placenta. NK cells comprise  $\sim 15\%$  of all lymphocytes and are defined phenotypically by expression of CD56 and lack of expression of CD3 on the cell surface (Robertson and Ritz, 1990). The majority ( $\sim 90\%$ ) of human NK cells have low density expression of CD56 (CD56<sup>dim</sup>) and express high levels of Fc $\gamma$  receptor IIIa (Fc $\gamma$ RIIIa; CD16<sup>+</sup>) whereas  $\sim 10\%$  of NK cells are CD56<sup>bright</sup>CD16<sup>dim</sup> or CD56<sup>bright</sup>CD16<sup>-</sup> (Cooper *et al.*, 2001). Uterine NK cells appear to be CD56<sup>bright</sup> and increase in number during the post-ovulatory luteal phase (King *et al.*, 1996).

A previous study by Beer *et al.* (1996) showed that an elevated percentage of peripheral blood NK cells was associated with recurrent failed IVF treatment cycles. Later, Fukui *et al.* (1999) showed that increased peripheral blood NK cell cytotoxicity level was associated with an increased rate of recurrent failed implantation after IVF treatment. More recent studies have confirmed elevated CD69 expression on NK cells as being associated with recurrent miscarriage and infertility of unknown aetiology (Ntrivalas *et al.*, 2001). Finally, a recent small non-randomized study by Coulam and Roussev (2003) revealed that infertile women undergoing IVF treatment also have a higher percentage of elevated CD69 expression on NK cells as compared to multiparous women.

CD69 belongs to the C-lectin type superfamily and is a type II integral membrane protein consisting of a disulphide-linked

homodimer with two phosphorylated chains (Ziegler *et al.*, 1993). It is a functional triggering molecule on activated NK cells and is one of the earliest cell surface activation markers expressed (Yokoyama, 1999). It is capable of inducing cytotoxicity and stimulating cytokine production (Zingoni *et al.*, 2000). Besides mediating NK cell cytotoxicity, it also mediates other NK cell functions such as proliferation, tumour necrosis factor (TNF- $\alpha$ ) production and expression of other activation antigens (Borrego *et al.*, 1999; Pisegna *et al.*, 2002).

CD94 is an inhibitory marker of NK cell function. It is part of the killing inhibitory receptor (KIR) family which is a sub-group of the C type lectin superfamily (Lopez-Botet *et al.*, 1997). Borrego *et al.* (1999) demonstrated that NK cell cytotoxicity could be blocked by the CD94 inhibitory receptor. Previous studies have shown that imbalances in CD69 and CD94 expression on NK cell could result in infertility of unknown aetiology or recurrent miscarriage (Ntrivalas *et al.*, 2001; Coulam *et al.*, 2003).

CD16 (also classified as Fc $\gamma$ RIIIa) is one of the low affinity receptors for the Fc region of IgG. Fc $\gamma$ RIIIa is an integral membrane protein expressed on NK cells, on a subset of T lymphocytes, and on a subpopulation of monocytes and macrophages (Ravetch and Perussia, 1989). Previously Fukui *et al.* (1999) showed that an increased percentage of peripheral blood CD16<sup>+</sup> NK cells was associated with failed implantation after IVF treatment.

The aim of this study was to document any association between the absolute count of activation marker (CD69), IgG Fc receptor (CD16) and inhibitor marker (CD94) expressing NK cells on the implantation and miscarriage rate after IVF treatment.

## Materials and methods

### Study population

From December 2002 to July 2003, 138 patients undergoing IVF treatment cycles were recruited into the study. Independent ethical approval was obtained from the Local Research Ethics Committee. Exclusion criteria: women with known immunological disease (anti-phospholipid antibodies, lupus anticoagulant, anticardiolipin antibodies), uterine abnormality (fibroid, uterine polyp, uterine septum), fewer than two embryos available for transfer or endometrium thickness <8 mm before embryo transfer. All transvaginal ultrasound scans were performed by a sonographer and exclusion of candidates was performed without the knowledge of the NK cell blood test result. Blood samples were obtained on the day of vaginal oocyte collection prior to the procedure. Informed consent was provided by all subjects at recruitment.

### Stimulation protocol

Pituitary down-regulation was achieved with either nafarelin or busarelin at mid-luteal phase. Ovarian stimulation was carried out with either recombinant FSH, hMG or urinary FSH. When follicles reached pre-ovulatory size (18–22 mm), 10 000 IU of hCG was administered. Oocytes were aspirated using transvaginal ultrasound guidance 34–36 h after hCG administration. All embryos were allowed to cleave and the best two or three embryos were selected for transfer. Embryo transfer was performed on day 2 or 3 using a

soft catheter (Wallace) with transabdominal ultrasound guidance. Progesterone supplement for luteal support (Cyclogest; Shire Pharmaceuticals Ltd, UK), 400 mg once a day *per vaginum* or *per rectum*, was commenced 1 day before embryo transfer and continued until a pregnancy test was performed 2 weeks after embryo transfer.

### Flow cytometric NK activation and inhibition quantification assay

Peripheral blood was collected in heparinized tubes and analysed within 24 h. Fifty millilitres of blood was placed in flow cytometric tubes (Becton Dickinson) and each incubated for 15 min at room temperature with mouse anti-human CD16–fluorescein isothiocyanate (FITC), anti-CD56 phycoerythrin (PE) (BD PharMingen), anti-CD3 PE Cy5 (Quest Biomedical), together with CD69 or CD94 APC (BD PharMingen) monoclonal antibodies (mAb). Isotypic control mAb included mouse IgG<sub>1</sub> FITC, IgG<sub>1</sub> APC, IgG<sub>1</sub> PE (BD PharMingen) and IgG<sub>1</sub> PE–Cy5 (Quest Biomedical). In this lyse, no wash procedure, 1 ml of Quicklysis lysing solution (Quest Biomedical) was added to each tube and incubated for a further 10 min at room temperature. Fifty millilitres of PerfectCount beads (Quest Biomedical) were then accurately pipetted to each tube and samples run with BD FACSCalibur flow cytometer. Cells negatively staining for CD3, but positively for CD56, were selected and their CD69 and CD94 expression analysed using a Cell Quest software (BD) using a four-colour protocol.

### Data analysis

All IVF data were collected in Medical System for IVF (Medical-Sys, UK) and analysed by Statistics Package for Social Sciences (SPSS, UK). Descriptive statistical analysis was performed initially to examine the normal distribution of all continuous variances for parametric statistical tests. The *t*-test was then used to compare the mean value in two groups: pregnant and not pregnant. Receiver operating characteristic (ROC) curve and area under curve (AUC) analysis were performed. The ROC curve represents the probability of true positive results (sensitivity) as a function of the probability of false positive results (1 - specificity). The AUC is a measure of the accuracy of a test. In order to perform the ROC curve calculation, set threshold values for the state variable (CD69 absolute count) were selected. These are arbitrary values selected to analyse the association of treatment outcome. For each CD69 threshold, separate curves were produced for treatment outcome and pregnancy outcome, making a total of 14 curves.  $\chi^2$  cross-tabulation test was used to analyse the significance of differences in pregnancy rates, miscarriage rates and live birth rates between groups. Analysis of variance was then conducted to assess the duration and amount of gonadotrophin required to achieve follicular maturity, estradiol levels on hCG day, number of mature follicles, number of available embryos for transfer, number of oocytes collected and fertilization rate between groups.

## Results

Of the 138 women who underwent IVF, 12 were excluded from statistical analysis. Of these 12, four had failed fertilization, four had only one embryo available for transfer, one had ovarian hyperstimulation syndrome and therefore did not have embryo transfer, two had an endometrial thickness <7.5 mm and one woman had poor quality embryos. None of the women who participated in the study were excluded due to abnormal uterine anatomy or known previous abnormal immunological tests.

**Table I.** Patients' demographics and stimulation characteristic between pregnant and non-pregnant women

	Non-pregnant	Pregnant	P-value
No. of patients	75	51	NA
Age (years) (mean ± SD)	35.45 ± 3.8	34.16 ± 4.0	NS
Tubal factor (%)	23.7	23.5	NS
Male factor (%)	25.0	27.6	NS
Other <sup>a</sup> (%)	16.3	15.6	NS
Unexplained (%)	35.0	33.3	NS
Duration of infertility (years) (mean ± SD)	4.60 ± 2.7	3.57 ± 2.3	NS
Basal FSH levels (IU/l) (mean ± SD)	7.82 ± 2.9	7.71 ± 3.8	NS
Mean no. of previous failed IVF attempts	1.88	1.37	NS
Mean No. of previous miscarriages	0.25	0.33	NS
Gonadotrophin <sup>b</sup> (IU)	3067.0	2531.6	NS
Estradiol (IU) on hCG day	8599.87	7015.88	NS
No. of oocytes collected (mean ± SD)	12.3 ± 5.5	12.9 ± 6.6	NS
Fertilization rate (%)	66.7	66.5	NS
No. of available embryos for transfer (mean ± SD)	7.96 ± 4.4	8.76 ± 5.7	NS
Mean no. of embryos transferred	2.18	2.04	NS

<sup>a</sup>Anovulation and endometriosis.

<sup>b</sup>Mean amount of gonadotrophin used for stimulation in IU (recombinant FSH, hMG or urinary FSH).

NA = not applicable; NS = difference not statistically significant ( $P > 0.05$ ).

Table I shows patients' treatment outcome, mean age, causes of infertility, duration of infertility, basal FSH levels, mean number of previous IVF attempts, number of previous miscarriages and outcome of ovarian stimulation in the pregnant and non-pregnant groups. There were no significant differences between the two groups with regard to any of the parameters.

Table II examines the relationship between the absolute count of CD69, CD94 and CD16 expressing CD56<sup>dim</sup> cells and the absolute count of CD69 and CD94 expressing CD56<sup>bright</sup> cells with IVF treatment outcome; pregnant versus non-pregnant. For women who had a successful ongoing pregnancy, the mean ( $\pm$ SD) value of the absolute CD56<sup>dim</sup>CD16<sup>+</sup>D69<sup>+</sup> NK cells was  $0.61 \times 10^6/l \pm 0.31 \times 10^6$ . For those women who failed to achieve pregnancy, the mean value of the absolute count of

**Table II.** Natural killer cell sub-population and CD69 expression between pregnant and non-pregnant women

	Non-pregnant	Pregnant	P-value
No. of patients	75	51	NA
CD56 <sup>dim</sup> CD16 <sup>+</sup> CD69 <sup>+</sup>	1.66 ± 0.52	0.61 ± 0.31	0.003
CD56 <sup>dim</sup> CD16 <sup>+</sup> CD94 <sup>+</sup>	142.1 ± 87	149.9 ± 117	NS
CD56 <sup>dim</sup> CD16 <sup>+</sup>	212.2 ± 149	233.0 ± 168	NS
CD56 <sup>bright</sup> CD69 <sup>+</sup>	0.31 ± 0.34	0.28 ± 0.31	NS
CD56 <sup>bright</sup> CD94 <sup>+</sup>	15.9 ± 8.9	16.9 ± 7.5	NS

Values are mean  $\pm$  SD absolute count ( $\times 10^6/l$ ) unless otherwise stated. NA = not applicable; NS = difference not statistically significant ( $P > 0.05$ ).

CD69-expressing NK cells was significantly higher at  $1.66 \times 10^6/l \pm 0.52 \times 10^6$  ( $P = 0.003$ ). The absolute count of CD94 and CD16 expressing CD56<sup>dim</sup> NK cells and CD56<sup>bright</sup> NK cells showed no significant difference between those women with successful and failed IVF treatment.

For the ROC analysis, 14 curves were produced, one for treatment outcome (pregnancy rate) and one for pregnancy outcome (live birth rate) for each of the seven chosen CD69 absolute count threshold. Table III illustrates the test characteristics and AUC for each selected CD69 absolute count threshold. The AUC values were maximum at a CD69 absolute count of  $1.0 \times 10^6/l$  for both treatment outcome (pregnancy rate) and pregnancy outcome (live birth rate). This indicated that  $1.0 \times 10^6/l$  is a better level for further statistical analysis as compared to the other selected levels. Increasing the threshold level improved specificity and positive predictive value at the expense of sensitivity. Therefore the threshold value of  $1.0 \times 10^6/l$  was selected for further statistical analysis without selecting a threshold level for the test.

The study population was then divided into two groups based on the absolute count of CD69-expressing NK cells. Group A women ( $n = 87$ ) had a count  $< 1.0 \times 10^6/l$  while group B women ( $n = 39$ ) had a count  $> 1.0 \times 10^6/l$ . Table IV shows number of women in each group, their mean age, causes of infertility, duration of infertility, basal FSH levels, mean number of previous IVF attempts and previous miscarriages, stimulation characteristics and treatment outcome in both groups. There were no significant differences between group A and group B with regard to age, causes and duration of infertility and basal FSH levels. The mean number of previous failed IVF attempts and the mean number of previous miscarriages were significantly higher in group B as compared to group A. There was no significant difference between the two groups with regard to amount of gonadotrophin used for stimulation, estradiol levels on hCG day, number of oocytes collected, fertilization rate, number of available embryos for transfer or number of embryos transferred. In group B, the implantation rate, pregnancy rate and live birth rate were significantly lower, and the miscarriage rate was significantly higher as compared to group A.

## Discussion

CD69 is one of the earliest specific activation markers expressed during large granulated lymphocyte activation, which includes the NK cell (Craston *et al.*, 1997; Marzio *et al.*, 1999; Llera *et al.*, 2001). Activated CD69<sup>+</sup> NK cells will release cytokines which will further activate other NK cells and the cellular immune system (Marzio *et al.*, 1999). Previous studies have also shown that elevated CD69 expression on NK cells is associated with an increase in cytotoxicity of NK cells towards target cells, which induces target cell lysis (Lanier *et al.*, 1988; De Maria *et al.*, 1994). Chao *et al.* (1999) have suggested that maternal NK cell CD69 expression is involved in recognition of HLA-G and HLA-C on the allogeneic embryonic and trophoblast cell surface. In theory, recognition of HLA-G and HLA-C expression is

**Table III.** The test characteristics and area under the curve (AUC) for each selected CD69 absolute count threshold

CD69 threshold ( $\times 10^6/l$ )	Outcome parameter per treatment cycle	AUC	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
0.7	Pregnancy rate	0.593	51.3	67.3	70.4	47.8
	Live birth rate	0.614	50.6	72.2	81.1	38.2
0.8	Pregnancy rate	0.614	47.3	75.5	74.5	48.7
	Live birth rate	0.652	47.1	83.3	87.0	40.0
0.9	Pregnancy rate	0.604	43.2	77.6	74.4	47.5
	Live birth rate	0.628	42.3	83.3	85.7	38.0
1.0	Pregnancy rate	0.635	40.0	82.4	77.8	48.3
	Live birth rate	0.667	40.9	92.1	92.3	40.2
1.1	Pregnancy rate	0.625	39.2	81.3	76.6	48.9
	Live birth rate	0.660	37.6	91.8	91.7	39.1
1.2	Pregnancy rate	0.618	29.4	89.8	83.3	47.3
	Live birth rate	0.651	32.9	97.2	96.5	38.0
1.3	Pregnancy rate	0.625	31.1	83.3	88.5	47.4
	Live birth rate	0.633	29.4	97.2	96.1	36.8

AUC = area under the ROC (receiver operating characteristic) curve; PPV = positive predictive value; NPV = negative predictive value

thought to protect the embryo from destruction by NK cells (Ellis *et al.*, 1989; Kovats *et al.*, 1990; King *et al.*, 1997). Ho *et al.* (1996) showed that NK cell cytotoxicity is decreased in a normal healthy pregnancy compared with an embryonic pregnancy. He suggested that activated NK cells, with CD69 expression on their cell surface, play an important role in the control of trophoblast growth and placental development. In support of this, *in vitro* models show that activated CD69 positive NK cells are capable of lysing trophoblasts (Helige *et al.*, 2001; Avril *et al.*, 2003).

In this study, we explored the relationship between the IVF treatment outcome with the quantification of activation receptor (CD69) and inhibitory receptor (CD94) on peripheral blood NK cells. Our results revealed that women who failed to achieve a pregnancy after IVF treatment have a

significantly higher level of activated CD56<sup>dim</sup>CD16<sup>+</sup>CD69<sup>+</sup> NK cells in the peripheral blood. This was evident despite the fact that there were no significant differences in patients' demographic details, number of previous failed IVF attempts or miscarriage, ovarian stimulation outcome, embryo quality or number of embryos transferred. This appears to be the first study to reveal elevated CD56<sup>dim</sup>CD16<sup>+</sup>CD69<sup>+</sup> peripheral blood NK cells in women who experience failed IVF treatment. It has however been reported that women with infertility needing IVF treatment and women who experience recurrent spontaneous miscarriage have significantly higher levels of peripheral CD56<sup>dim</sup>CD16<sup>+</sup>CD69<sup>+</sup> NK cells (Ntrivalas *et al.*, 2001; Coulam *et al.*, 2003). The mechanism of implantation and the precise role of NK cells in implantation are still not fully understood, but it can be speculated

**Table IV.** Patients' demographics, stimulation characteristic, treatment and pregnancy outcome in group A and B

	Group A CD69 $\leq 1.0 \times 10^6/l$	Group B CD69 $> 1.0 \times 10^6/l$	P-value
No. of patients	87	39	NA
Age (years) (mean $\pm$ SD)	35.54 $\pm$ 3.7	35.85 $\pm$ 4.6	NS
Tubal factor (%)	21.3	20.0	NS
Male factor (%)	22.3	30.0	NS
Other <sup>a</sup> (%)	17.0	12.5	NS
Unexplained (%)	39.4	37.5	NS
Duration of infertility (years) (mean $\pm$ SD)	4.11 $\pm$ 2.6	4.20 $\pm$ 2.5	NS
Basal FSH levels (mean $\pm$ SD)	8.12 $\pm$ 3.8	7.82 $\pm$ 3.3	NS
No. of previous failed IVF attempts (mean $\pm$ SD)	1.29 $\pm$ 1.5	2.60 $\pm$ 2.77	0.001
No. of previous miscarriages (mean $\pm$ SD)	0.20 $\pm$ 0.48	0.45 $\pm$ 0.75	0.023
Gonadotrophin <sup>b</sup> (IU) (mean $\pm$ SD)	2579.8 $\pm$ 979.1	3489.5 $\pm$ 576.7	NS
Estradiol (IU) on hCG day	8430.60	7727.84	NS
No. of oocytes collected (mean $\pm$ SD)	12.59 $\pm$ 6.0	11.67 $\pm$ 5.9	NS
Fertilization rate (%) (mean $\pm$ SD)	63.75 $\pm$ 22.7	70.49 $\pm$ 21.2	NS
No. of available embryos for transfer (mean $\pm$ SD)	8.16 $\pm$ 5.3	7.82 $\pm$ 4.0	NS
No. of embryos transferred (mean $\pm$ SD)	2.08 $\pm$ 0.37	2.15 $\pm$ 0.43	NS
Implantation rate, % ( <i>n</i> )	28.2 (53/188)	13.1 (11/84)	0.004
Pregnancy rate per cycle started, % ( <i>n</i> )	48.3 (42/87)	23.1 (9/39)	0.006
Live birth rate per cycle started, % ( <i>n</i> )	40.2 (35/87)	7.7 (3/39)	< 0.0001
Miscarriage rate, % ( <i>n</i> )	16.7 (7/42)	66.7 (6/9)	0.005

<sup>a</sup>Anovulation and endometriosis.

<sup>b</sup>Mean amount of gonadotrophin used for stimulation (recombinant FSH, hMG or urinary FSH).

NA = not applicable; NS = difference not statistically significant ( $P > 0.05$ ).

that an excess of activated CD69<sup>+</sup> NK cells might play a negative role in successful implantation.

We further evaluated CD56<sup>bright</sup>CD69<sup>+</sup> cells, a sub-population of NK cells, which are phenotypically similar to uterine NK cells. The level of peripheral CD56<sup>bright</sup>CD69<sup>+</sup> NK cells was not significantly different between the pregnant and non-pregnant groups. In contrast, Kodama *et al.* (1998) reported that increased numbers of CD56<sup>bright</sup>CD69<sup>+</sup> NK cells were found in the decidua in women with spontaneous miscarriage compared with normal pregnancies. This finding may suggest that although phenotypically similar, uterine CD56<sup>bright</sup> NK cells may not be related to peripheral blood CD56<sup>bright</sup> NK cells. Our study demonstrates that there is no association between the absolute count of CD56<sup>dim</sup>CD16<sup>+</sup>CD94<sup>+</sup> NK cells and CD56<sup>bright</sup>CD94<sup>+</sup> NK cells with the outcome of IVF treatment. However, further functional cytotoxicity studies would be needed to evaluate CD94 expression and cytotoxicity of NK cells. In contrast to a previous study by Fukui *et al.* (1999), the present study did not demonstrate any statistical difference in the absolute count of CD56<sup>dim</sup>CD16<sup>+</sup> NK cells and CD56<sup>bright</sup>CD16<sup>+</sup> NK cells between successful and failed IVF treatment. However, for statistical analysis these authors used percentage of CD16<sup>+</sup> NK cells instead of the absolute count, which may not be accurate, since the percentage can vary depending on the composition of other lymphocytes in a sample.

Further statistical analysis was carried out based on the absolute count of CD56<sup>dim</sup>CD16<sup>+</sup>CD69<sup>+</sup> NK cells above and below  $1.0 \times 10^6/l$  in order to examine other variables. The value of  $1.0 \times 10^6/l$  was selected for analysis after ROC testing. This study is not intended to establish a threshold level for the CD69 NK cell testing procedure. Estimating a threshold level and testing it in the same sample will create bias. The selected threshold value should be tested and its value confirmed or rejected in a separate study with a new set of samples. The result of the ROC analysis revealed that the sensitivity and negative predictive value were rather low, perhaps because there are numerous factors affecting IVF outcome. For example, a woman with a low CD69 count may not achieve a pregnancy because her embryos are genetically abnormal.

The results show that for those women with CD56<sup>dim</sup>CD16<sup>+</sup>CD69<sup>+</sup> NK cells  $>1.0 \times 10^6/l$ , the implantation rate and the pregnancy rate were significantly lower and the miscarriage rate was significantly higher as compared to women with CD56<sup>dim</sup>CD16<sup>+</sup>CD69<sup>+</sup> NK cells below this level. The combination of lower implantation rate and higher miscarriage rate result in a significant lower live birth rate. With this finding, we speculated that elevated peripheral activated NK cells may play a detrimental role in IVF treatment outcome. The data also reveal that women with elevated peripheral CD56<sup>dim</sup>CD16<sup>+</sup>CD69<sup>+</sup> NK cells had a significantly higher number of previous miscarriages and previous failed IVF treatments despite the fact that there were no statistically significant demographic differences between pregnant and non-pregnant groups (Table I). This finding is in accord with previous studies (Emmer *et al.*, 2000; Ntrivalas *et al.*, 2001) showing that levels of activated NK cells were raised in

women with spontaneous recurrent miscarriages. However, we cannot exclude the possibility that previous miscarriage and failed IVF treatment may be the cause of the elevated CD69<sup>+</sup> NK cell count, nor that the selected threshold value chosen may, by chance, divide the patients into two groups with very different clinical prognostic factors that determine the poorer outcome in the group with high CD69<sup>+</sup> NK cell count. This needs to be examined further in a new prospective study using logistic regression analysis adjusting for the impact of these clinical variables.

In conclusion, our data suggest that an elevated level of CD56<sup>dim</sup>CD16<sup>+</sup>CD69<sup>+</sup> peripheral blood NK cells is associated with a reduced implantation rate of embryos in IVF treatment. Those women with an elevated peripheral blood CD56<sup>dim</sup>CD16<sup>+</sup>CD69<sup>+</sup> NK cell count who achieve a pregnancy after IVF manifest a significantly higher miscarriage rate. A high level of peripheral blood CD69<sup>+</sup> NK cells may play a negative role in IVF treatment outcome but this has to be explored in a new prospective study.

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