An increase in the absolute count of CD56$^{\text{dim}}$CD16$^+$CD69$^+$ NK cells in the peripheral blood is associated with a poorer IVF treatment and pregnancy outcome

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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$^+$ (dim + bright) and CD3$^{-}$ by flow cytometry. The absolute counts of the CD69$^+$, CD16$^+$ and CD94$^+$ expressing NK cells were recorded and their relation to IVF treatment outcome and miscarriage rate was analysed. RESULTS: The mean (±SD) absolute count of the CD56$^{\text{dim}}$CD16$^+$CD69$^+$ NK cells for women who had a successful ongoing pregnancy was 0.61 $\times$ 10$^6$/l (± 0.31). For those women who failed to achieve a pregnancy, the mean value of the absolute count of CD56$^{\text{dim}}$CD16$^+$CD69$^+$ NK cells was significantly ($P = 0.003$) higher at 1.66 $\times$ 10$^6$/l (± 0.52). The absolute count of CD56$^{\text{dim}}$CD16$^+$CD69$^+$ and CD56$^{\text{dim}}$CD16$^+$CD69$^+$ 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$^{\text{dim}}$CD16$^+$CD94$^+$ expressing NK cell count of > 1.0 $\times$ 10$^5$/l compared to women with count below this value (IR 28.2% and MR 16.7%). Further analysis of the absolute count of CD56$^{\text{bright}}$CD69$^+$ and CD56$^{\text{bright}}$CD94$^+$ 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$^{\text{dim}}$CD16$^+$CD69$^+$) in the peripheral blood is associated with a reduced rate of embryo implantation in IVF treatment. Furthermore, women with high CD56$^{\text{dim}}$CD16$^+$CD69$^+$ 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 ~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 (~90%) of human NK cells have low density expression of CD56 (CD56$^{\text{dim}}$) and express high levels of Fcγ receptor IIIa (FcγRIIIa; CD16$^+$) whereas ~10% of NK cells are CD56$^{\text{bright}}$CD16$^{\text{dim}}$ or CD56$^{\text{bright}}$CD16$^+$ (Cooper et al., 2001). Uterine NK cells appear to be CD56$^{\text{bright}}$ 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 cyto-
toxicity and stimulating cytokine production (Zingoni et al., 2000). Besides mediating NK cell cytotoxicity, it also medi-
ates other NK cell functions such as proliferation, tumour
necrosis factor (TNF-α) 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 recep-
tor. 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 FcyRIIIa) is one of the low affi-
nity receptors for the Fc region of IgG. FcyRIIIa is an inte-
mbrane 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 peri-
pheral blood CD16+ 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 anti-
odies), tumour necrosis factor (TNF-α) production and expression of other
activation antigens (Borrego et al., 1999; Pisegna et al.,
2002).

From December 2002 to July 2003, 138 patients undergoing IVF
treatment. 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 Phar-
aceuticals 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 isothio-
cyanate (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 IgG1 FITC, IgG1 APC, IgG1 PE (BD PharMingen) and IgG1 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 calcu-
lation, 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, sepa-
rate 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 fertiliza-
tion, 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 abnor-
mal immunological tests.

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

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

^Amenotulation and endometriosis.
^bMean 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 II. Natural killer cell sub-population and CD69 expression between pregnant and non-pregnant women

<table>
<thead>
<tr>
<th></th>
<th>Non-pregnant</th>
<th>Pregnant</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>75</td>
<td>51</td>
<td>NA</td>
</tr>
<tr>
<td>CD56dimCD16+CD69^+</td>
<td>1.66 ± 0.52</td>
<td>0.61 ± 0.31</td>
<td>0.003</td>
</tr>
<tr>
<td>CD56dimCD16+CD94^+</td>
<td>142.1 ± 87</td>
<td>149.9 ± 117</td>
<td>NS</td>
</tr>
<tr>
<td>CD56^+CD16^+</td>
<td>212.2 ± 149</td>
<td>233.0 ± 168</td>
<td>NS</td>
</tr>
<tr>
<td>CD56brightCD69^+</td>
<td>0.31 ± 0.34</td>
<td>0.28 ± 0.31</td>
<td>NS</td>
</tr>
<tr>
<td>CD56brightCD94^+</td>
<td>15.9 ± 8.9</td>
<td>16.9 ± 7.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean ± SD absolute count (× 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 × 10^6/l ± 0.52 × 10^6 (P = 0.003). The absolute count of CD94 and CD16 expressing CD56^dim NK cells and CD56^bright 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 × 10^6/l for both treatment outcome (pregnancy rate) and pregnancy outcome (live birth rate). This indicated that 1.0 × 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 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 × 10^6/l while group B women (n = 39) had a count > 1.0 × 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^+ 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
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 anembryonic pregnancy. He suggested that activated NK cells, with CD69 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 CD56dimCD16+CD69+ 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 CD56dimCD16+CD69+ 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 CD56dimCD16+CD69+ 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 III. The test characteristics and area under the curve (AUC) for each selected CD69 absolute count threshold

<table>
<thead>
<tr>
<th>CD69 threshold (×10⁶/l)</th>
<th>Outcome parameter per treatment cycle</th>
<th>AUC</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.7</td>
<td>Pregnancy rate</td>
<td>0.593</td>
<td>51.3</td>
<td>67.3</td>
<td>70.4</td>
<td>47.8</td>
</tr>
<tr>
<td></td>
<td>Live birth rate</td>
<td>0.614</td>
<td>50.6</td>
<td>72.2</td>
<td>81.1</td>
<td>38.2</td>
</tr>
<tr>
<td>0.8</td>
<td>Pregnancy rate</td>
<td>0.614</td>
<td>47.3</td>
<td>75.5</td>
<td>74.5</td>
<td>48.7</td>
</tr>
<tr>
<td></td>
<td>Live birth rate</td>
<td>0.652</td>
<td>47.1</td>
<td>83.3</td>
<td>87.0</td>
<td>40.0</td>
</tr>
<tr>
<td>0.9</td>
<td>Pregnancy rate</td>
<td>0.604</td>
<td>43.2</td>
<td>77.6</td>
<td>74.4</td>
<td>47.5</td>
</tr>
<tr>
<td></td>
<td>Live birth rate</td>
<td>0.628</td>
<td>42.3</td>
<td>83.3</td>
<td>85.7</td>
<td>38.0</td>
</tr>
<tr>
<td>1.0</td>
<td>Pregnancy rate</td>
<td>0.635</td>
<td>40.0</td>
<td>82.4</td>
<td>77.8</td>
<td>48.3</td>
</tr>
<tr>
<td></td>
<td>Live birth rate</td>
<td>0.667</td>
<td>40.9</td>
<td>92.1</td>
<td>92.3</td>
<td>40.2</td>
</tr>
<tr>
<td>1.1</td>
<td>Pregnancy rate</td>
<td>0.625</td>
<td>39.2</td>
<td>81.3</td>
<td>76.6</td>
<td>48.9</td>
</tr>
<tr>
<td></td>
<td>Live birth rate</td>
<td>0.660</td>
<td>37.6</td>
<td>91.8</td>
<td>91.7</td>
<td>39.1</td>
</tr>
<tr>
<td>1.2</td>
<td>Pregnancy rate</td>
<td>0.618</td>
<td>29.4</td>
<td>89.8</td>
<td>83.3</td>
<td>47.3</td>
</tr>
<tr>
<td></td>
<td>Live birth rate</td>
<td>0.651</td>
<td>32.9</td>
<td>97.2</td>
<td>96.5</td>
<td>38.0</td>
</tr>
<tr>
<td>1.3</td>
<td>Pregnancy rate</td>
<td>0.625</td>
<td>31.1</td>
<td>83.3</td>
<td>88.5</td>
<td>47.4</td>
</tr>
<tr>
<td></td>
<td>Live birth rate</td>
<td>0.633</td>
<td>29.4</td>
<td>97.2</td>
<td>96.1</td>
<td>36.8</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th></th>
<th>Group A CD69 ≤ 1.0 × 10⁶/l</th>
<th>Group B CD69 &gt; 1.0 × 10⁶/l</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>87</td>
<td>39</td>
<td>NA</td>
</tr>
<tr>
<td>Age (years) (mean ± SD)</td>
<td>35.54 ± 3.7</td>
<td>35.85 ± 4.6</td>
<td>NS</td>
</tr>
<tr>
<td>Tubal factor (%)</td>
<td>21.3</td>
<td>20.0</td>
<td>NS</td>
</tr>
<tr>
<td>Male factor (%)</td>
<td>22.3</td>
<td>30.0</td>
<td>NS</td>
</tr>
<tr>
<td>Other (%)</td>
<td>17.0</td>
<td>12.5</td>
<td>NS</td>
</tr>
<tr>
<td>Unexplained (%)</td>
<td>39.4</td>
<td>37.5</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of infertility (years) (mean ± SD)</td>
<td>4.11 ± 2.6</td>
<td>4.20 ± 2.5</td>
<td>NS</td>
</tr>
<tr>
<td>Basal FSH levels (mean ± SD)</td>
<td>8.12 ± 3.8</td>
<td>7.82 ± 3.3</td>
<td>NS</td>
</tr>
<tr>
<td>No. of previous failed IVF attempts (mean ± SD)</td>
<td>1.29 ± 1.5</td>
<td>2.60 ± 2.77</td>
<td>0.001</td>
</tr>
<tr>
<td>No. of previous miscarriages (mean ± SD)</td>
<td>0.20 ± 0.48</td>
<td>0.45 ± 0.75</td>
<td>0.023</td>
</tr>
<tr>
<td>Gonadotrophin⁵ (IU) (mean ± SD)</td>
<td>2579.8 ± 979.1</td>
<td>3489.5 ± 576.7</td>
<td>NS</td>
</tr>
<tr>
<td>Estradiol (IU) on hCG day</td>
<td>8430.60</td>
<td>7727.84</td>
<td>NS</td>
</tr>
<tr>
<td>No. of oocytes collected (mean ± SD)</td>
<td>12.59 ± 6.0</td>
<td>11.67 ± 5.9</td>
<td>NS</td>
</tr>
<tr>
<td>Fertilization rate (%) (mean ± SD)</td>
<td>63.75 ± 22.7</td>
<td>70.49 ± 21.2</td>
<td>NS</td>
</tr>
<tr>
<td>No. of available embryos for transfer (mean ± SD)</td>
<td>8.16 ± 5.3</td>
<td>7.82 ± 4.0</td>
<td>NS</td>
</tr>
<tr>
<td>No. of embryos transferred (mean ± SD)</td>
<td>2.08 ± 0.37</td>
<td>2.15 ± 0.43</td>
<td>NS</td>
</tr>
<tr>
<td>Implantation rate, % (n)</td>
<td>28.2 (53/188)</td>
<td>13.1 (11/84)</td>
<td>0.004</td>
</tr>
<tr>
<td>Pregnancy rate per cycle started, % (n)</td>
<td>48.3 (42/87)</td>
<td>23.1 (9/39)</td>
<td>0.006</td>
</tr>
<tr>
<td>Live birth rate per cycle started, % (n)</td>
<td>40.2 (35/87)</td>
<td>7.7 (3/39)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Miscarriage rate, % (n)</td>
<td>16.7 (7/42)</td>
<td>66.7 (69/101)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

aAnovulation and endometriosis.

bMean 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⁺ NK cells might play a negative role in successful implantation. We further evaluated CD56brightCD69⁺ cells, a subpopulation of NK cells, which are phenotypically similar to uterine NK cells. The level of peripheral CD56brightCD69⁺ NK cells was not significantly different between the pregnant and non-pregnant groups. In contrast, Kodama et al. (1998) reported that increased numbers of CD56brightCD69⁺ 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 CD56bright NK cells may not be related to peripheral blood CD56bright NK cells. Our study demonstrates that there is no association between the absolute count of CD56dimCD16⁺ CD94⁺ NK cells and CD56brightCD94⁺ 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 CD56dimCD16⁺ NK cells and CD56brightCD16⁺ NK cells between successful and failed IVF treatment. However, for statistical analysis these authors used percentage of CD16⁺ 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 CD56dimCD16⁺CD69⁺ NK cells above and below 1.0 × 10⁶/l in order to examine other variables. The value of 1.0 × 10⁶/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 CD56dimCD16⁺D69⁺ NK cells > 1.0 × 10⁶/l, the implantation rate and the pregnancy rate were significantly lower and the miscarriage rate was significantly higher as compared to women with CD56dimCD16⁺D69⁺ 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 CD56dimCD16⁺D69⁺ 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 1). 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⁺ 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⁺ 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 CD56dimCD16⁺CD69⁺ peripheral blood NK cells is associated with a reduced implantation rate of embryos in IVF treatment. Those women with an elevated peripheral blood CD56dimCD16⁺CD69⁺ NK cell count who achieve a pregnancy after IVF manifest a significantly higher miscarriage rate. A high level of peripheral blood CD69⁺ NK cells may play a negative role in IVF treatment outcome but this has to be explored in a new prospective study.

References


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