

# Simple enumerations of peripheral blood natural killer (CD56<sup>+</sup> NK) cells, B cells and T cells have no predictive value in IVF treatment outcome

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**BACKGROUND:** To evaluate the association between the absolute counts of the peripheral natural killer (NK) cells (including total CD56<sup>+</sup> NK cells, CD56<sup>dim</sup> NK cells and CD56<sup>bright</sup> NK cells), B cells and T cells on the implantation rate and miscarriage rate after IVF treatment. **METHODS:** This was a prospective observation study. A total of 138 patients who underwent IVF treatment from December 2002 to July 2003 were recruited to the study. Blood samples were obtained on the day of vaginal oocyte retrieval prior to the procedure. The absolute counts of lymphocytes, NK cells, B cells and T cells were identified by flow cytometry. These absolute counts and their relationships to IVF treatment outcome and miscarriage rate were analysed. **RESULTS:** There were no significant differences with regard the mean values of absolute lymphocyte count, T cell count, B cell count and NK cell count (including total CD56<sup>+</sup> NK, CD56<sup>dim</sup> NK and CD56<sup>bright</sup> NK cells) between the pregnant and non-pregnant groups and also between the ongoing pregnancy and miscarriage groups. The cause of infertility, duration of infertility, basal FSH levels, number of previous failed IVF treatments, number of previous miscarriages and stimulation characteristics were not significantly different between the pregnant and non-pregnant groups. Previous studies have suggested that women with a history of recurrent miscarriage and those with infertility accompanied by recurrent failed IVF treatments are associated with a peripheral blood NK cell percentage >12%, therefore further analysis of peripheral CD56<sup>+</sup> NK cell levels <12% (group A) and >12% (group B) was performed. There was no significant difference in implantation rate (group A: 17.0%; group B: 23.2%), pregnancy rate (group A: 36.6%; group B: 47.7%) or miscarriage rate (group A: 23.3%; group B: 28.6%). **CONCLUSION:** There were no significant differences between simple enumerations of peripheral blood NK cells (including total CD56<sup>+</sup> NK, CD56<sup>dim</sup> NK and CD56<sup>bright</sup> NK cells), B cells and T cells with IVF treatment outcome and pregnancy outcome. Women who had a peripheral NK cell level >12% did not have higher number of previous pregnancy losses. Importantly their pregnancy rate was not reduced and their miscarriages were not increased compared to women who had a peripheral NK cells level <12%.

*Key words:* B cells/CD56/IVF/natural killer cells/T cells

## Introduction

For the conceptus to implant and for pregnancy to be maintained, the conceptus has to protect itself from the maternal immune system (Hill *et al.*, 1992; Yokoyama *et al.*, 1994). This involves a balance between maternal immune defence mechanisms and invasion by the allogenic trophoblast. Lymphocytes including NK cells, T cells and to a lesser extent B cells are clearly present in the human decidua (Bulmer and Sunderland, 1984) and the most abundant amongst these in the endometrium is the NK cell (Moffett-King, 2002). All lymphocytes are, however, in intimate direct contact with trophoblast cells and have the potential to initiate an immune response (Lachapelle *et al.*, 1996). Yamada *et al.*

(1994) showed that trophoblasts can stimulate peripheral blood mononuclear cells to proliferate and produce embryotoxic factors in women with recurrent miscarriage. Others have suggested that NK cells, monocytes/macrophages, and T cells could produce decidual toxic factors resulting in fetal loss (Baines and Gendron, 1990; Clark, 1994). While the factors controlling the influx, proliferation and differentiation of these cells in the uterus are unknown, the processes seem to be dependent on decidualization of the stromal cells.

Previous studies have suggested that an increase in circulating lymphocytes might be associated with recurrent miscarriages. Thus Souza *et al.* (2002) reported that an increase

in peripheral blood absolute T cell count, but not NK cell count, was evident in women with recurrent abortion. Furthermore, Yamamoto *et al.* (1999) reported that the peripheral blood NK cell percentage was not significantly different between pregnant women and women who had a miscarriage with a chromosomally normal or abnormal pregnancy, while Yamada *et al.* (2001) reported that NK cell activity was only higher in women who had a miscarriage with a chromosomally normal pregnancy. In the same publication, Yamada *et al.* (2001) also reported that the peripheral NK cell percentages were not significantly different between women who had miscarriage with a chromosomally normal or abnormal pregnancy. However, in a later publication (Yamada *et al.*, 2003) the same group reported that both NK cell activity and percentage were elevated in women who suffered from recurrent miscarriage and had a miscarriage with a chromosomally normal pregnancy. Coulam *et al.* (1995) also suggested that the peripheral blood NK cell percentage could predict pregnancy outcome. Additionally, Beer *et al.* (1996) showed that women with a history of recurrent miscarriage and infertile women with recurrent failed IVF treatments were associated with a peripheral blood NK cell percentage >12%. These findings have not been replicated by any other research groups. However, numerous practitioners are offering immunomodulation therapy based on the evidence provided by Coulam *et al.* (1995) and Beer *et al.* (1996) that the peripheral blood NK cell absolute count or percentage can affect IVF or pregnancy outcome.

The objective of this study was to investigate, prospectively, women undergoing IVF treatment to determine whether there was any association between the peripheral blood NK cell (including total CD56<sup>+</sup> NK, CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells), T cell and B cell percentages and absolute counts and implantation success or miscarriage in patients undergoing IVF treatment.

## Methods and materials

### Study population

From December 2002 to July 2003, 138 patients undergoing IVF treatment cycles were recruited into the study. Independent approval was obtained from the Local Research Ethics Committee and informed consent was obtained from each patient prior to the study. Ten millilitres of peripheral blood were obtained on the day of vaginal oocyte retrieval prior to the procedure. Exclusion criteria were: women with known immunological disease (antiphospholipid 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. Exclusion of candidates was performed without the knowledge of the blood test result. Blood samples were obtained on the day of vaginal oocyte collection prior to the procedure. We had noted that Giuliani *et al.* (1998) reported that controlled ovarian stimulation in assisted reproduction did not affect the immune system.

### Stimulation protocol for IVF treatment

Stimulation protocol for IVF treatment was as previously described (Thum *et al.*, 2003). Briefly, pituitary down-regulation was achieved with either nafarelin or buserelin 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 day 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 vagina or per rectum, was commenced 1 day before embryo transfer and continued until a pregnancy test was performed. A pregnancy test was performed 2 weeks after embryo transfer.

### Flow cytometric NK activation and inhibition quantification assay

Ten millilitres of peripheral blood was collected in heparinized tubes and analysed within 24 h. Fifty millilitres of each sample was incubated for 15 min at room temperature with 10 ml mouse anti-CD56 PE (BD PharMingen), anti-CD3 PE Cy5 (Quest Biomedical) monoclonal antibodies (mAb). Isotypic control mAb included mouse 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. A volume of 50 ml of PerfectCount beads (Quest Biomedical) was then accurately pipetted to each tube and samples run with BD FACSCalibur flow cytometer.

### Data analysis

All IVF data were collected prospectively in Medical System for IVF (MedicalSys, 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 variables for parametric statistical tests. Analysis of variance was then conducted to assess the duration and amount of gonadotrophin required to achieve follicular maturity, number of mature follicles, number of available embryos for transfer, number of oocytes collected, fertilization rate, the absolute count of lymphocytes and its subpopulation (NK cells, B cells and T cells) between the pregnant and non-pregnant women after IVF treatment. The  $\chi^2$  cross-tabulation test was used to analyse for difference of pregnancy rates, miscarriage rates and live birth rates between women with NK cell percentage >12% and <12% as suggested by Beer *et al.* (1996).

## Results

During the study period, from December 2002 to July 2003, 138 women who underwent IVF were recruited. Twelve women were excluded from statistical analysis; four had

failed fertilization, four had only one embryo available for transfer, one had OHSS therefore did not have embryo transfers, two had an endometrial thickness <7.5 mm and one had poor quality embryos. The overall pregnancy rate was 38.6%, the implantation rate 19.1% and the miscarriage rate 24.5% for all the patients recruited. Out of 128 cycles, 82 (64.1%) were conventional IVF and 46 (35.9%) were IVF in conjunction with ICSI. There were no differences regarding ovarian stimulation characteristics and cycle outcomes between conventional IVF and IVF with ICSI.

There were no significant differences between the two groups with regard to patients' characteristics (Table I).

Table II confirms the absence of any significant differences in absolute lymphocyte count, T Cell, B cell, CD56<sup>+</sup> NK cell, CD56<sup>dim</sup> and CD56<sup>bright</sup> NK cells in the pregnant and non-pregnant groups. This was also the case for those women with ongoing pregnancy and miscarriage (Table III).

Table IV examines the relationship between outcome of ovarian stimulation, IVF treatment outcome and pregnancy outcome with CD56<sup>+</sup> NK cell percentage equal to or <12%

and >12%. There were no significant differences with regard to the outcome of ovarian stimulation, IVF treatment outcome and pregnancy outcome between the two groups. The pregnancy rate and miscarriage rate were higher in CD56<sup>+</sup> NK cell >12% group but not significantly so.

**Discussion**

Maternal immunological mechanisms have been postulated to explain recurrent miscarriage, unexplained infertility and recurrent failed IVF cycles (Norwitz *et al.*, 2001). A successful pregnancy depends on protection or down-regulation of potentially harmful maternal immune responses at the utero-placental interface to enable a functional placenta to develop (King *et al.*, 1997). Although implantation and development and maintenance of the placenta are typically localized processes, it is interesting to evaluate whether any systemic immunological changes can have a detrimental effect on implantation, placentation or on the conceptus. However, it is difficult to examine the local environment at the implantation site. Clearly a biopsy of the endometrium cannot be performed near to the time of implantation or before embryo

**Table I.** Patients' demographic and stimulation characteristics

	Not pregnant	Pregnant
No. of patients	75	51
Age (years)	35.45 ± 3.8	34.16 ± 4.0
Tubal factor (%)	23.7	23.5
Male factor (%)	25.0	27.6
Other <sup>a</sup> (%)	16.3	15.6
Unexplained (%)	35.0	33.3
Duration of infertility in years (mean ± SD)	4.60 ± 2.7	3.57 ± 2.3
Basal FSH level (IU/l) (mean ± SD)	7.82 ± 2.9	7.71 ± 3.8
Mean no. of previous failed IVF attempts	1.88	1.37
No. of previous miscarriages (± SD)	0.25	0.33
Gonadotrophin <sup>b</sup> (IU)	3067.0	2531.6
Estradiol (IU) on hCG day	8599.87	7015.88
No. of oocytes collected (mean ± SD)	12.3 ± 5.5	12.9 ± 6.6
Fertilization rate (%)	66.7	66.5
Embryos available for transfer (mean ± SD)	7.96 ± 4.4	8.76 ± 5.7
Mean no. of embryos transferred	2.18	2.04

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

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

No differences between groups were statistically significant (*P* > 0.05).

**Table II.** The absolute counts of lymphocyte, T-cell, B-cell, NK cell and its sub-groups between pregnant and not pregnant groups

	Non-pregnant	Pregnant
No. of patients	75	51
Lymphocyte absolute count (× 10 <sup>9</sup> ) <sup>a</sup>	2.23 ± 0.76	2.07 ± 0.61
T lymphocyte absolute count (× 10 <sup>9</sup> ) <sup>a</sup> (%)	1.62 ± 0.53 (73.1)	1.54 ± 0.50 (74.2)
B lymphocyte absolute count (× 10 <sup>9</sup> ) <sup>a</sup> (%)	0.38 ± 0.02 (16.2)	0.29 ± 0.04 (13.7)
CD56 <sup>+</sup> NK cell absolute count (× 10 <sup>9</sup> ) <sup>a</sup> (%)	0.232 ± 0.02 (10.5)	0.256 ± 0.01 (11.8)
CD56 <sup>dim</sup> NK cell absolute count (× 10 <sup>9</sup> ) <sup>a</sup> (%)	0.211 ± 0.07 (9.7)	0.238 ± 0.03 (10.9)
CD56 <sup>bright</sup> NK cell absolute count (× 10 <sup>9</sup> ) <sup>a</sup> (%)	0.017 ± 0.008 (0.82)	0.018 ± 0.007 (0.95)

<sup>a</sup>Mean ± SD.

No differences between groups were statistically significant (*P* > 0.05).

**Table III.** The absolute counts of lymphocyte, T-cell, B-cell, NK cell and its sub-groups between on going pregnancy and miscarriage groups

	Ongoing pregnancy	Miscarriage
No. of patients	38	13
Lymphocyte absolute count (× 10 <sup>9</sup> ) <sup>a</sup>	2.05 ± 0.6	2.13 ± 0.6
T lymphocyte absolute count (× 10 <sup>9</sup> ) <sup>a</sup> (%)	1.54 ± 0.5 (74.7)	1.53 ± 0.4 (72.4)
B lymphocyte absolute count (× 10 <sup>9</sup> ) <sup>a</sup> (%)	0.267 ± 0.01 (12.8)	0.34 ± 0.02 (16.1)
CD56 <sup>+</sup> NK cell absolute count (× 10 <sup>9</sup> ) <sup>a</sup> (%)	0.243 ± 0.02 (11.9)	0.249 ± 0.02 (11.4)
CD56 <sup>dim</sup> NK cell absolute count (× 10 <sup>9</sup> ) <sup>a</sup> (%)	0.224 ± 0.01 (10.9)	0.231 ± 0.02 (10.6)
CD56 <sup>bright</sup> NK cell absolute count (× 10 <sup>9</sup> ) <sup>a</sup> (%)	0.018 ± 0.008 (1.0)	0.017 ± 0.007 (0.8)

<sup>a</sup>Mean ± SD.

No differences between groups were statistically significant (*P* > 0.05).

**Table IV.** Stimulation characteristics and cycle outcomes

	Group A CD56 <sup>+</sup> NK cell ≤ 12%	Group B CD56 <sup>+</sup> NK cell > 12%
No. of patients	82	44
Gonadotrophin (IU) <sup>a</sup>	2902.2	2590.3
No. of oocytes collected (mean ± SD)	12.32 ± 5.9	12.27 ± 6.0
Fertilization rate (%)	64.15 ± 23.5	67.8 ± 22.9
Embryos available for transfer (mean ± SD)	7.75 ± 4.8	8.39 ± 5.2
Mean no. of embryos transferred	2.07	2.16
Implantation rate (%)	17.0	23.2
Pregnancy rate per cycle started (%)	36.6 (30/82)	47.7 (21/44)
Live birth rate per cycle started (%)	28.1 (23/82)	34.1 (15/44)
Miscarriage rate (%)	23.3 (7/30)	28.6 (6/21)

<sup>a</sup>Mean amount of gonadotrophin used for stimulation.

No differences between groups were statistically significant (*P* > 0.05).

transfer during IVF treatment. Moreover, a biopsy prior to the treatment cycle/pregnancy may not reflect the condition at the time of implantation. In consequence it is difficult to evaluate the local immunological milieu in this situation. The objective of the study was to evaluate whether variations in the absolute numbers of different lymphocyte subsets had a detrimental effect on implantation, placentation or on the conceptus after IVF treatment.

The results of this study showed no significant differences in the peripheral T cell count between women with a positive or negative IVF treatment outcome or with pregnancy outcome, i.e. live birth and miscarriage. This finding is in accord with previous studies. Thus Quenby *et al.* (1999) showed no significant difference in the endometrial T cell count between women with recurrent miscarriage and multiparous women with no history of recurrent miscarriage. Additionally, Vassiliadou and Bulmer (1998) showed similar number of T cells in normal first trimester deciduas compared to deciduas obtained after spontaneous miscarriage. However, Yamada *et al.* (1994) showed that peripheral blood T cells from women with recurrent miscarriage were sensitive to trophoblasts when cultured *in vitro* and were able to proliferate and produce embryotoxic factors. However, this sensitivity may be the result of previous miscarriages rather than the cause, as even non-viable pregnancy tissue can initiate a maternal immune response. Regarding peripheral blood B cells, these were also not significantly different between pregnant and non-pregnant women after IVF treatment or between women with ongoing pregnancy and miscarriage. This finding is in keeping with Quenby *et al.* (1999), who revealed that endometrial B cells were not significantly different between women with a history of recurrent miscarriage and multiparous women with no history of recurrent miscarriage. These findings suggest that variation in peripheral blood T cell and B cell counts have no significant influence on IVF treatment outcome or pregnancy outcome.

In this study, we explored the relationship between the IVF treatment outcome, pregnancy outcome and peripheral blood CD56<sup>+</sup> NK cell values. Our results revealed the absence of any significant relationship between peripheral blood CD56<sup>+</sup> NK cell values (absolute count and percentage) and IVF treatment outcome or pregnancy outcome. Therefore, an increase in the peripheral blood CD56<sup>+</sup> NK cell percentage or absolute count may not be associated with increased failed implantation or an increased rate of miscarriage. This finding is in accord with previous studies (Yamamoto *et al.*, 1999; Michimata *et al.*, 2002) in showing that the peripheral blood NK cell percentage has no association with miscarriages and no predictive value for pregnancy outcome. Our finding is in contrast with that of Coulam *et al.* (1995), where the authors suggested that the peripheral blood NK cell percentage could predict pregnancy outcome. However, the flow cytometric method used to identify NK cells in the Coulam study may not have been accurate due to the lack of CD3 antigen assessment to exclude CD56 expressing T cells. Consequently the lymphocytes examined were a mixture of T cells and NK cells; therefore one could argue that their conclusion that the

peripheral blood NK cell percentage could predict pregnancy outcome may not be accurate. Moreover, the study group used by Coulam *et al.* (1995) was not homogeneous and included women receiving donor oocytes or intravenous immunoglobulin G (IV IgG) treatment. In our study we analysed both the absolute and percentage lymphocyte subset counts with a particular focus on the absolute count. It is well known that the latter is more accurate than the percentage, as the percentage is heavily influenced by the presence of the other lymphocyte subsets in a sample. Our finding is also in contrast with that of Yamada *et al.* (2003), where the authors reported that NK cell percentage was elevated in women who suffered from recurrent miscarriage and had a miscarriage with a chromosomally normal pregnancy. However, the statistical analysis in Yamada's study included women with known endocrine or autoimmune disorders.

We further evaluated the IVF outcome and pregnancy outcome for patients with peripheral blood CD56<sup>+</sup> NK cell percentages <12% and ≥12%. This subdivision of the study group with a 12% threshold for analysis was based on the work by Beer *et al.* (1996). They reported that women with peripheral blood CD56<sup>+</sup> NK cell percentages >12% had a reduced pregnancy rate after IVF treatment and increased miscarriage risk. This work has encouraged many practitioners to offer immunomodulation therapy to patients, including paternal lymphocyte immunization, i.v. IgG and anti-tumour necrosis factor (TNF) therapy, based on the percentage of their peripheral blood NK cells. The results of our study revealed no significant difference with regard to pregnancy or miscarriage rate in patients with peripheral blood CD56<sup>+</sup> NK cell percentages <12% or ≥12%. Therefore, our data suggest that the 12% threshold has no predictive value in IVF treatment outcome and risk miscarriage. It is important to consider some gaps in the work reported by Beer *et al.* (1996). Thus it was unclear at what stage the blood samples were obtained from the study subjects. This is clearly important as the results of NK analysis can vary according to the point in a treatment cycle at which the sample was obtained. Furthermore, part of their analysis compared the NK cell percentage in pregnant women with non-pregnant women having a history of recurrent miscarriage. This comparison may also not be valid as lymphocyte composition can vary during a pregnancy. There was also no explanation of how the arbitrary threshold level of 12% was selected. Our finding suggests that the peripheral blood NK cell count and percentage have no bearing on IVF treatment outcome, pregnancy outcome or risk of miscarriage. More importantly the 12% arbitrary threshold has no predictive value in determining IVF treatment outcome and risk of miscarriage. Finally, it is worth noting that the NK cell percentage range in a healthy individual can be ≥20% (Cooper *et al.*, 2001). Therefore based on the findings of our study it may be inappropriate to offer immunomodulation therapy for this group of patients. Beer *et al.* (1996), however, reported that no women with CD56<sup>+</sup> NK cells >18% had delivered a live-born child. This is in contrast to the results of our study in which 15 women with CD56<sup>+</sup> NK cells >18% completed

an IVF treatment cycle and six (40%) became pregnant. Of these six women, five delivered and one woman had a miscarriage. This gives a live birth rate of 30% and a miscarriage rate of 20% for this group of women.

In conclusion, our data suggest that there is no significant association between simple enumerations of peripheral blood NK cells (including total CD56<sup>+</sup> NK, CD56<sup>dim</sup> NK and CD56<sup>bright</sup> NK cells), B cells and T cells with IVF treatment outcome and pregnancy outcome. Women who have a peripheral NK cell level >12% do not have either a reduced rate of achieving pregnancy or higher rate of pregnancy loss after IVF treatment. Even with a markedly elevated peripheral blood CD56<sup>+</sup> NK cell of >18%, there was no association with poorer IVF treatment outcome or pregnancy outcome.

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