ORIGINAL ARTICLE

Detailed Analysis of Peripheral Blood Natural Killer Cells in Women with Repeated IVF Failure

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Problem
To analyse the peripheral blood NK cells in women with repeated IVF failure (RIF) and a fertile control group to determine which parameters best differentiate the two populations.

Methods
Peripheral blood from the luteal phase of 171 women with RIF and 33 fertile controls was analysed by four-colour flow cytometry for NK cell concentration, subset differentiation and the activation marker CD69.

Results
Women with RIF had significantly increased NK cell numbers as determined by concentration ($P < 0.05$) and percentage of lymphocytes ($P < 0.001$), increased concentration of the $CD56^{dim}$ subtype ($P < 0.05$), and increased concentration of activated $CD56^{dim}$ $CD69^{+}$ cells ($P = 0.0001$). There was no correlation between any NK cell parameters with the length of infertility or number of embryo transfer cycles.

Conclusions
Peripheral blood NK cell activity is significantly higher in women with RIF than in fertile controls. Future trials of immune therapy in women undergoing IVF should target those with high NK activity.

Introduction

Infertility is an increasing medical and social problem. Delayed childbearing exposes couples to increased risk of causes of infertility including endometriosis, pelvic infection, and poor egg and sperm quality. Many infertile couples turn to IVF, which can address most problems with some success and has become the treatment of choice, funded to some degree in many healthcare systems. However, in spite of significant advances over the last three decades, IVF cycle success rates are universally <50% and often <25%.[1] Medically, this results in considerable morbidity as couples have to face the prospect of repeated cycles; socio-economically, it is often not only the individual couples, but taxpayers who have to pay the bill for repeated IVF failure through healthcare funding. There is still no formal consensus definition for repeated IVF failure (RIF), although suggestions have included the failure of at least three cycles of IVF or two cycles of IVF plus two cycles using frozen embryos.[2] What is clear, though, is the pressing need to manage couples with RIF effectively and in particular to continue to try to improve IVF outcomes.

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The assessment of peripheral blood NK cells in women with RIF was popularized by Beer et al.[3] claim that elevated numbers of peripheral blood natural killer cells or higher levels of natural killer activity are predictors of pregnancy loss. This finding has been supported by a number of further studies including assessment of NK cell numbers,[4,5] activation status by CD69[6,7] and the chromium-51 assay.[7–10] Other studies have concluded that while levels may be higher, they are not predictive of outcome.[11]

Thus, the role of blood NK cell testing is still unclear, and further research is needed.[12] We have developed a peripheral blood test for natural killer cell analysis by flow cytometry, which is robust and reliable. In a detailed assessment of women with recurrent miscarriage, we demonstrated that this test identified 15% of women as having high blood NK cell levels as a proportion of lymphocytes and high levels of activation as expressed by the CD69 surface marker.[13] The purpose of this study was to perform a similar analysis in women with RIF, comparing those women with a control group with no infertility history. It was expected that the prevalence of NK cell abnormalities would be lower than in the recurrent miscarriage population, given the many unrelated factors contributing to IVF success and infertility.

Materials and methods

Study Population

Peripheral blood testing for NK cell analysis was established at St George Hospital, Kogarah, specifically for the purpose of assessing women with repeated implantation failure. Between September 2005 and May 2009, 570 assays were performed of which 171 satisfied the study definition of RIF (≥ 3 failed embryo transfer cycles, including both fresh and frozen embryos). The definition of RIF required at least three transfer cycles with a negative pregnancy test. Known biochemical pregnancies were defined as miscarriages and women with recurrent miscarriage as their primary diagnosis were excluded. The control population was recruited from the hospital community of women who had no previous history of reproductive problems. All tests were performed in the mid-luteal phase, excluding women who were pregnant or on hormonal contraception.

Sample Collection and Flow Cytometry Analysis

Peripheral blood samples were drawn between the hours of 9am–12pm and were analysed on site by four-colour flow cytometry within four hours of being taken. Prior to staining for surface marker analysis, a white cell count (WCC) and lymphocyte percentage was obtained from the Sysmex XE2100 Haematology analyser (Roche Diagnostics, Castle Hill, NSW, Australia). Four tubes were set up per patient using the monoclonal antibodies to CD45 PerCP, CD3 APC, CD19 APC, CD56 PE, CD69 FITC (Becton Dickinson, San Jose, CA, USA), CD14 PE and CD16 FITC (Immunotech, Marseilles, France). Isotype and fluorochrome matched controls (Becton Dickinson) were used as negative controls. Antibodies were used at the volumes recommended by the manufacturer and incubated for 10 min with a volume of peripheral blood to give a concentration of 1×10^6 white cells per tube. Cells were then lysed and fixed with FACS lysing solution (Becton Dickinson) for 15 min before being washed three times prior to analysis. Flow cytometric analysis was performed on the Becton Dickinson FACSCalibur dual laser flow cytometer (488/633 nm), using CellQuest software (Becton Dickinson) (Fig. 1). 2000 NK cells (CD3 negative, CD56 positive) were collected for NK subset determination and 10,000 NK cells for measurement of activation using CD69. Total NK cells and the $CD56^{+Dim}$ subset were expressed as a percentage of lymphocytes as well as absolute number. The activated ($CD69^+$) $CD56^{+Dim}$ subset was expressed as a percentage of $CD56^{+Dim}$ cells as well as an absolute number.

Statistical Analysis

GraphPad Prism software (San Diego, CA, USA) was used to analyse the results. Mann–Whitney tests were used to compare the continuous variables. ROC analyses were performed to determine the sensitivity and specificity, and correlation studies were used to determine the relationship between NK cell parameters. ANOVA tests were used to conduct subgroup analysis and Dunnett's multiple comparison tests were used as post-tests.

Results

Women in the RIF group had infertility for a mean of 3.9 years, with causes of infertility cited as male

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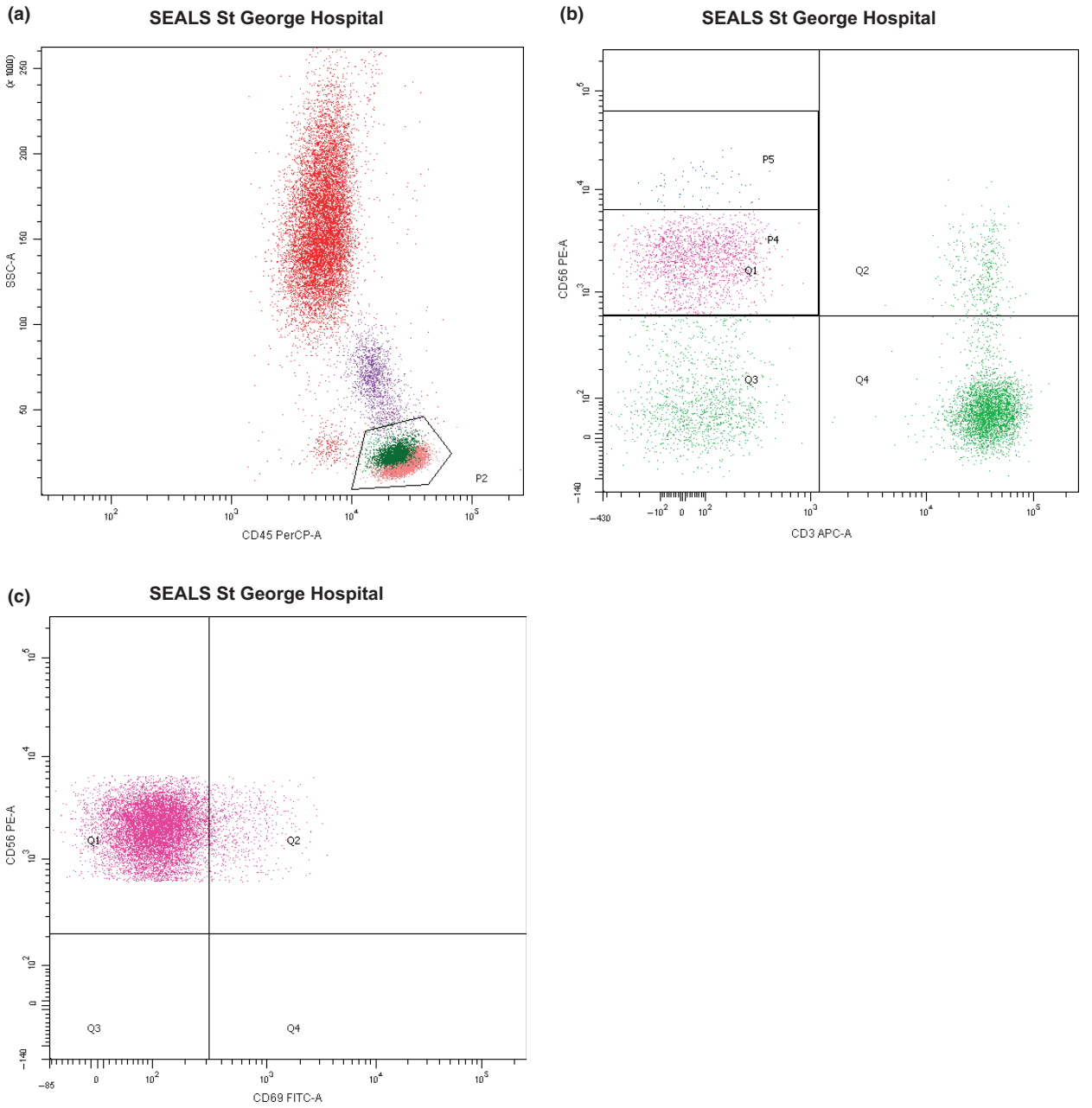

Fig. 1 Flow cytometry dot plots. (a) Side scatter versus CD45 (white blood cell marker). Lymphocytes are gated. (b) Staining for CD3 and CD56. NK cells are CD3- and $CD56^{+}$. NK cell subtypes are gated with $CD56^{dim}$ in pink and $CD56^{bright}$ in blue. (c) NK cell activation as assessed by the CD69 marker.

factor (28%), tubal (10%), pelvic endometriosis or adhesions (28%), ovulatory (12.8%), uterine fibroids or septum (10.5%), and unexplained (28%). They were significantly older than the control population ($P = 0.03$), had experienced more miscarriages ($P < 0.0001$) and fewer live births ($P < 0.0001$) (Table I). Women with RIF had a mean of three egg collections, 5.7 embryo transfer procedures and 7.3 embryos transferred (including fresh and frozen) (Table I). A third of embryos transferred were day 5 blastocysts, and the remainder day 2/3 embryos. The RIF group had a higher prevalence of autoimmune

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Table I Demographic Data

Cohort	Outcome	Age (years)	Pregnancies (no.)	Livebirths (no.)	Miscarriages (no.)	Length of infertility (years)	Embryo transfer procedures (no.)
RIF	*n*	171	171	171	171	171	171
	Mean	37.58	1.6	0.18	1.5	3.9	5.7
	S.D.	4.94	1.51	0.46	1.43	2.50	3.06
	Median	38	1	0	1	3	5
	Range	25–50	0–7	0–2	0–6	0.5–20	3–18
Control	*n*	33	33	33	33		
	Mean	34.39	1.6	1.21	0.3		
	S.D.	7.14	1.82	1.32	0.68		
	Median	36	1	1	0		
	Range	20–47	0–7	0–5	0–2		
P (Mann–Whitney)		<0.05	n.s.	<0.0001	<0.0001		

RIF, repeated IVF failure.

diseases (12.8% versus 0%), most commonly Hashimoto's thyroiditis.

Women with RIF had significantly increased peripheral blood NK cell activity as detected by number of NK cells as a percentage of total peripheral lymphocytes, NK cell concentration, $CD56^{dim}$ concentration, number of CD56 dim $CD69^{+}$ cells as a percentage of total CD56 dim cells and CD56 dim $CD69^{+}$ concentration (Table II).

The relationship between CD56 subgroups was assessed by the CD56 bright/dim ratio. Although the number of $CD56^{dim}$ cells as a percentage of total NK cells was higher in the RIF population, and the ratio of $CD56^{bright}$ to $CD56^{dim}$ NK cells was lower, neither

Table II Detailed NK Cell Analysis in Women with RIF Versus Controls

Cohort	Variable	%NK	NK Conc ($\times 10^9$/L)	% Dim NK	Dim NK Conc ($\times 10^9$/L)	Bright/Dim (ratio)	%CD69 Dim	$CD69^{Dim}$ Conc ($\times 10^6$/L)
RIF	*n*	171	171	171	171	171	171	171
	Mean	11.33	0.23	94.46	0.22	0.063	4.35	8.61
	S.D.	4.90	0.11	3.78	0.11	0.046	7.88	14.91
	Median	10.77	0.21	95.19	0.2	0.05	2.96	5.82
	Range	3.57–29.84	0.005–0.65	79.89–99.48	0.05–0.64	0–0.25	0.54–96.26	1.31–181.7
Control	*n*	33	33	33	33	33	33	33
	Mean	8.73	0.20	92.57	0.19	0.09	2.77	4.52
	S.D.	5.30	0.13	6.34	0.13	0.87	1.87	3.26
	Median	7.47	0.17	94.38	0.16	0.04	2.4	3.76
	Range	3.53–33.95	0.06–0.73	69.69–99.68	0.05–0.73	0.01–0.45	0.58–8.93	0.97–15.83
P (Mann–Whitney)		<0.001	<0.05	n.s	<0.05	n.s.	<0.01	0.0001

RIF, repeated IVF failure.
%NK = Total NK percentage = NK concentration/Total peripheral lymphocytes ×100.
NK conc = Total NK concentration = NK count × 10^9/L.
% Dim NK = $CD56^{+Dim}$ Cell percentage = $CD56^{+Dim}$ NK concentration/NK concentration × 100.
Dim NK Conc = $CD56^{+Dim}$ NK concentration = $CD56^{+Dim}$ NK Count × 10^9/L.
Bright/Dim ratio = $CD56^{+Bright}$/$CD56^{+Dim}$ ratio = $CD56^{+Bright}$ NK concentration/$CD56^{+Dim}$ concentration.
%CD69 Dim = Percentage of activated ($CD60^{+}$) $CD56^{+Dim}$ NK cells = $CD69^{+}CD56^{+Dim}$ concentration/$CD56^{+Dim}$ concentration × 100.
CD69 Dim Conc = $CD69^{+}CD56^{+Dim}$ NK cell concentration = $CD69^{+}CD56^{+Dim}$ count × 10^6/L.

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difference reached statistical significance (Table II). There were no significant differences in $CD56^{bright}$ percentage, concentration or CD69 expression (not shown).

A receiver operator characteristic (ROC) analysis (Fig. 2) comparing NK% in the RIF and control cohorts was performed (AUC = 0.6793, $P = 0.001$). Using a NK percentage of 18% as a cut-off, the test was able to discriminate between the RIF and control cohorts with a sensitivity of 11.05% and a specificity of 97.06%, giving a likelihood ratio of 3.76. In other words, this test identified 11% of women with RIF with high NK cell levels.

Further ROC analysis (Fig. 3) comparing $CD56^{dim}$ $CD69^{+}$ concentrations in the RIF and control cohorts revealed that this parameter was better able to discriminate between the RIF and control populations than NK percentage (AUC = 0.7254, $P = 0.001$). Using a $CD56^{dim}$ $CD69^{+}$ concentration of 12×10^{6}/mL as a cut-off, the test is able to discriminate between the RIF and control cohorts with a sensitivity of 13% and specificity of 97.06%, giving a likelihood ratio of 4.39.

A Pearson's correlation test found a weak positive association between NK percentages and concentration of $CD56^{dim}$ $CD69^{+}$ in the RIF population ($r = 0.3409$, $P < 0.0001$, $r^2 = 0.1162$).

Women in the RIF cohort were separated into subgroups based on the length of infertility (Table III) and number of previous embryo transfer cycles (Table IV). There were no significant differences in any of the subgroups for either NK cell percentage or $CD69^{+}$ concentration (not shown). Some women in this study had repeated blood tests, either for confirmation of the result or following a longer period of time (in some cases following successful pregnancy). Over a range of 1–42 months, 42 women had repeated testing, and there was no significant difference in either NK cell percentage or

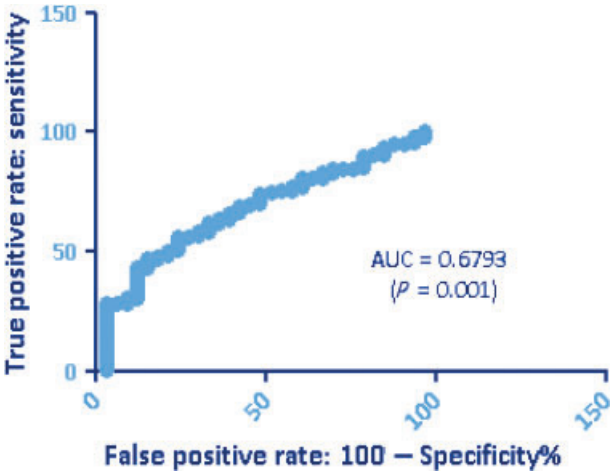

Fig. 2 Receiver-operated characteristic analysis of NK percentages in the RIF population compared with controls.

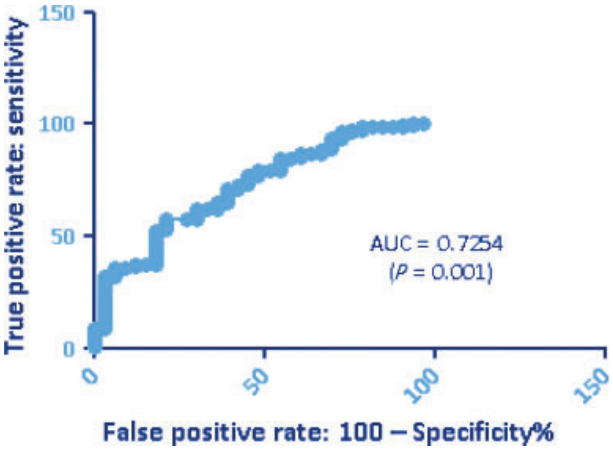

Fig. 3 Receiver-operated characteristic analysis of $CD56^{dim}$ $CD69^{+}$ concentrations in the RIF population compared with controls.

Table III Length of Infertility and NK Cell Percentage of Lymphocytes

	0.5–2 years	2.5–3.5 years	4–5 years	6–20 years
N (number of women)	44	42	51	24
Mean	10.74	11.60	11.46	11.11
Median	10.14	11.39	10.94	10.55
Kruskal–Wallis $P = 0.8137$				

Table IV Number of Previous Embryo Transfer Cycles and NK Cell Percentage

	0–2	3	4–5	6–18
N (number of women)	1	49	51	70
Mean	10.11	11.63	11.10	11.12
Median	7.960	11.08	10.34	10.48
Kruskall–Wallis $P = 0.5477$				

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Table V Women with Repeated Blood Tests: NK Cell Percentage and CD69 Concentration

	%NK-1 (n = 42)	%NK-2 (n = 42)	CD69Conc-1 (n = 35)	CD69Conc-2 (n = 35)
Median	13.53	12.46	8.0	7.9
Range	5.06–29.84	5.26–25.68	2.3–105	3–81.9
Wilcoxon matched pairs test	P = 0.1133		P = 0.8731	

%NK-1 = NK cell percentage on first blood test.
%NK-2 = NK cell percentage on second blood test.
CD69Conc-1 = NK cell CD69-positive cells concentration on first blood test.
CD69Conc-2 = NK cell CD69-positive cells concentration on second blood test.
Blood tests were taken at a median of 7.5 months apart (range, 1–42 months).

NK cell CD69 concentration in the paired samples (Table V). Using a cut-off value of 18% for total NK percentage (as defined above), repeated testing confirmed a diagnosis above or below that threshold in 38 of 42 (90.5%) women.

Eighty-six women with RIF had at least one other abnormality detected in a comprehensive screen including parental karyotype, thyroid function, insulin resistance, thrombophilia and autoantibody screening. The most common abnormality was the presence of anticardiolipin antibodies (ACAs), which were detected in 44 women. There was no significant difference in NK cell parameters between women who were ACA positive and negative, or for any other subgroup (although numbers were much smaller).

Discussion

This study has demonstrated that women with RIF have altered peripheral blood NK cell parameters compared with normal fertile controls. In particular, NK cell numbers as a percentage of lymphocytes were over 18% in 11% of women with RIF, which was significantly higher than the control group and supports previous studies.[3,5,10] It is not surprising, and indeed, it is reassuring that the test had a low sensitivity (11%) as many women with RIF are likely to have multiple problems or may simply be unlucky with embryo genetic quality. The test is an ineffective tool to distinguish women who may develop RIF from a general population, but an effective test in differentiating subgroups within the RIF population.

One study has reported no difference in NK cell $CD56^{dim}$ or $CD56^{bright}$ concentrations between successful and unsuccessful patients with IVF.[11] Whilst that study concluded that NK testing is not worthwhile, it should be noted that subjects included all women undergoing IVF rather than those with RIF as in our study. Given that our study suggests that only 11% of women with RIF have high NK cell levels, it is likely that numbers were too small to cause a difference in that population. Furthermore, NK cell analysis may have been significantly affected (and differences further blunted) by samples being taken during the IVF cycle, and by the analysis being carried out by a laboratory off-site. It is often underappreciated that flow cytometry for NK cells is far from an automated test, and blood handling, consistent laboratory technique and interlaboratory differences may influence outcomes reported.[12]

We have shown that blood NK cells from women with RIF have higher expression of the activation marker CD69, as we have previously reported in women with recurrent miscarriage.[13] This has also been previously reported in women with primary unexplained infertility,[6] in infertile women undergoing IVF,[7] and is associated with a reduced rate of IVF success.[14] Using a ROC analysis, we concluded that a $CD56^{dim}$ $CD69^{+}$ concentration of over 12×10^6/mL was able to discriminate between patients with RIF and controls, with high specificity of 97.06%. There was a weak association between NK percentages and $CD56^{dim}$ $CD69^{+}$ concentration, although only 11.62% of the variance between the two data sets was explained by this correlation (r^2 = 0.1162), suggesting that these two parameters should be considered as potentially independent markers of raised peripheral blood NK cell activity.

We believe that this study is the first to attempt to assess blood NK cell status with length of infertility or previous IVF history. In establishing the reliability of our NK cell blood test, we noted that women having repeat blood tests in different cycles, in some

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cases months or even years apart, had remarkably consistent NK cell parameters (Table V). In the main study of women with RIF, individuals were not studied longitudinally; however, we also found no differences in any NK cell parameters according to increasing length of infertility or increasing number of embryo transfer cycles. Numbers in the subgroups may have been too small to demonstrate any changes, but it is also possible that NK cell levels may represent a constant feature of immune function, and the women with high levels could have been identified by testing prior to their failed IVF cycles. In our control population, only 3% had high levels, and this gives an estimate for the likely pickup rate of high levels in infertile women contemplating IVF of between 3 and 11%. If this is true, many women may wish to be tested prior to starting IVF to consider whether additional immune therapy may be beneficial.

Immune therapy is recommended on the basis that certain treatments, namely intravenous immunoglobulin (IVIg), TNF-α inhibitors and corticosteroids, are all able to suppress NK cell levels.[15] However, results thus far have been inconclusive, with differences in target, intervention, study design, patient inclusion and outcome measured all accounting for at least some of the conflicting evidence.[16,17] IVIg has been shown to reduce peripheral NK cell levels,[18] or cytotoxicity[19] which may be of some benefit for women such as those included in our trial, although meta-analysis reviews (in studies not targeting NK cell analysis) have found that there is no proven benefit to patients with IVF taking IVIg.[20] Similarly, prednisolone can suppress NK cell cytotoxicity *in vitro*,[21] and we[22] and others[23,24] have reported the cases of women with multiple repeated implantation failure who achieved a successful pregnancy with prednisolone. Prednisolone use has been aimed primarily at women with otherwise unexplained recurrent miscarriage and is the immunosuppressant of choice for many clinicians, owing to its relative safety, price, ease of administration and minimal side effects.[12,13] Despite the controversy of NK cell testing, and any immune therapy prescribed on the basis of these results, we believe that this study provides justification for a large-scale clinical trial to investigate the benefit of immune suppression in women with raised blood NK cells and RIF.

The results of the current study must be interpreted with caution as it was a retrospective study and so cannot establish a causal link between elevated NK cell parameters and RIF, but merely an association. It should be noted that while the control population had no past or current infertility diagnosis, potential future infertility could not be excluded. A prospective study would be required to account for such possible bias. The study was also prone to bias as the RIF population was older than the controls, and there was no control for this and other factors that are known to affect peripheral blood NK cell levels and activation status, including age,[25] time of the menstrual cycle,[26,27] exercise[28,29] and stress.[30] However, it is noteworthy that within the RIF group, there was no difference between NK cell parameters in younger women ($\leq$38 years, $n = 90$) and older women (>38 years, $n = 81$) (data not shown). This lends support to the proposition that high NK cell levels are a genuine feature of an individual's immunological function as discussed earlier.

Another criticism of blood NK cell testing is the apparently broad reference range for NK cell parameters in some population studies (5–30%),[31] making any attempt to define an abnormally 'high' level within that range meaningless. However, population-based studies have included men (known to have significantly higher levels than women) and not excluded women with reproductive failure. Simply excluding men produces a range remarkably similar to our findings that a 'high' level is defined as over 18%.[13]

It is still not known how (or indeed if) altered peripheral blood NK cell activity can affect embryo implantation. While embryo quality (primarily genetic) is almost certainly the most significant factor determining IVF success, the development of the endometrium is also crucial. Indeed, recent research has highlighted the interactions between the embryo and the endometrium and indicated that the endometrium of some women may have an abnormal 'receptivity' profile.[32] This may be of particular importance in RIF, where it is likely that a genetically normal embryo is transferred in some cycles.

The mechanism of embryo implantation is still poorly understood. Uterine natural killer (NK) cells are by far the most common immune cell-type present at the site of implantation and are the most likely candidates that control the process from the maternal interface. Uterine NK cell analysis is difficult in terms of both the invasiveness of the sampling procedure and the laboratory assessment.[33] We have presented preliminary data indicating a likely correlation between blood and uterine NK cell

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numbers,[34] which has been confirmed by others.[35] It is also possible (though as yet unproven) that an altered NK cell CD56 dim/bright subtype ratio leads to the alteration in the subtypes present in the uterus.[36] This could impact on implantation by effects on cytokine production for example.

We also argue that the network of the immune system means that multiple interactions are likely to cause the overall effect. In other words, blood test abnormalities may not in themselves cause implantation failure, but may still reflect immunological dysfunction at the implantation site. In terms of assessing NK cells, much debate has been concerned with the fact that the majority of peripheral blood NK cells are of a different phenotype (with different functions) to uterine NK cells and hence of no relevance.[15,31] Such debate, while crucial for our overall understanding, has little clinical value. The reality is that a blood test is far preferable for patients than an endometrial biopsy, and therefore, the analysis of peripheral blood NK cells in women with RIF deserves assessment in its own right.

Conclusions

Women with RIF have significantly altered NK cell parameters when compared with fertile controls. The concentration of $CD56^{dim}$ $CD69^{+}$ cells was the best discriminating test, and 13% of women with RIF had elevated concentrations of $CD56^{dim}$ $CD69^{+}$ cells ($>12 \times 10^{6}$/mL). This supports our hypothesis that if NK cell abnormalities do indicate an immune implantation disorder, it is likely to represent an imbalance of activated NK cell subsets, rather than the absolute numbers of NK cells.

While the peripheral blood test for NK cells is able to define a subgroup of women, it is still unclear whether this is a clinically useful thing to do. It was not the place of this paper to discuss clinical implications or therapy, although we believe that these baseline data provide a means to undertake clinical trials of immune suppressive therapy. We have preliminary data indicating that outcome is probably improved with immune therapy (unpublished study in progress), and we have also started a prospective randomized trial.

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References

1 Wang YA, Chambers GM, Dieng M, Sullivan EA: Assisted reproductive technology in Australia and New Zealand 2007. *In* Assisted Reproduction Technology Series No. 13. Cat. no. PER 47. Canberra, AIHW, 2009, p. 24.
2 Tuckerman E, Mariee N, Prakash A, Li TC, Laird S: Uterine natural killer cells in peri-implantation endometrium from women with repeated implantation failure after IVF. *J Reprod Immunol* 2010; 87:60–66.
3 Beer AE, Kwak J, Ruiz J: Immunophenotypic profiles of peripheral blood lymphocytes in women with recurrent pregnancy losses and in infertile women with multiple failed in vitro fertilization cycles. *Am J Reprod Immunol* 1996; 35:376–382.
4 Kaider AS, Kaider BD, Janowicz PB, Roussev RG: Immunologic evaluation in women with reproductive failure. *Am J Reprod Immunol* 1999; 42:335–346.
5 Michou VI, Kanavaros P, Athanassiou V, Chronis GB, Stabamas S, Tsilivakos V: Fraction of the peripheral blood concentration of CD56 + /CD16-/CD3- cells in total natural killer cells as an indication of fertility and infertility. *Fertil Steril* 2003; 80(Suppl. 2):691–697.
6 Ntrivalas EI, Kwak-Kim JYH, Gilman-Sachs A, Chung-Bang H, Ng SC, Beaman KD, et al.: Status of peripheral blood natural killer cells in women with recurrent spontaneous abortions and infertility of unknown aetiology. *Hum Reprod* 2001; 16:855–861.
7 Coulam C, Roussev R: Correlation of NK cell activation and inhibition markers with NK cytotoxicity among women experiencing immunologic implantation failure after in vitro fertilisation. *J Assist Reprod Genet* 2003; 20:58–62.
8 Aoki K, Kajiura S, Matsumoto Y, Ogasawara M, Okado S, Yagami Y, Gleicher N: Preconceptual natural-killer-cell activity as a predictor of miscarriage. *Lancet* 1995; 345:1340–1342.
9 Matsubayashi H, Shida M, Kondo A, Suzuki T, Sugi T, Izumi SI, et al.: Preconception peripheral natural killer cell activity as a predictor of pregnancy outcome in patients with unexplained infertility. *Am J Reprod Immunol* 2005; 53:126–131.
10 Fukui A, Fuji S, Yamagishi E, Kimura H, Sato S, Saito Y: Natural killer cell subpopulations and cytotoxicity for infertile patients undergoing in vitro fertilisation. *J Reprod Immunol* 1999; 41:413–422.
11 Thum M, Bhaskaran S, Bansal A, Shehata H, Ford BS, Abdalla H: Simple enumerations of peripheral blood natural killer (CD56 + NK) cells, B cells and T cells have no predictive value in IVF treatment outcome. *Hum Reprod* 2005; 20:1272–1276.
12 Sacks G. Natural killer cell analysis. *In* "How to Improve IVF Success Rates", G Kovacs (ed.). Cambridge, Cambridge University Press, 2011, pp 28–32.
13 King K, Smith S, Chapman M, Sacks G: Detailed analysis of peripheral blood natural killer cells in women with recurrent miscarriage. *Hum Reprod* 2010; 25:52–58.
14 Thum MY, Bhaskaran S, Abdalla HI, Ford B, Sumar N, Shehata H, et al.: An increase in the absolute count of CD56dimCD16 + CD69 + NK cells in the peripheral blood is associated with a poorer IVF treatment and pregnancy outcome. *Hum Reprod* 2004; 19:2395–2400.
15 Moffett A, Regan L, Braude P: Natural killer cells, miscarriage and infertility. *BMJ* 2004; 329:1283–1285.
16 Carp H, Toder V, Torchinsky AE: Allogenic leukocyte immunisation after five or more miscarriages. *Hum Reprod* 1997; 12:250–255.
17 Clark D, Coulam C, Daya S, Chaouat G: Unexplained sporadic and recurrent miscarriage in the new millennium: a critical analysis of

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immune mechanisms and treatments. *Hum Reprod Update* 2001; 7:501–511.
18 Morikawa M, Yamada H, Kato E, Shimada S, Kishida T, Yamada T, et al.: Massive intravenous immunoglobulin treatment in women with four or more recurrent spontaneous abortions of unexplained etiology: down-regulation of NK cell activity and subsets. *Am J Reprod Immunol* 2001; 46:399–404.
19 Kotlan B, Padanyl A, Batordi J, Fulop V, Szugetvari I, Rajczy K, Penzes M, Gyodi E, Reti M, Petranyl G: Alloimmune and autoimmune background in recurrent pregnancy loss – successful immunotherapy by intravenous immunoglobulin. *Am J Reprod Immunol* 2006; 55:331–340.
20 Porter T, LaCoursiere Y, Scott J: Immunotherapy for recurrent miscarriage. *Cochrane Database Syst Rev* 2006; 2:CD000112.
21 Thum M, Bhaskaran S, Abdalla H, Ford B, Sumar N, Bansal A: Prednisolone suppresses NK cell cytotoxicity in vitro in women with a history of infertility and elevated NK cell cytotoxicity. *Am J Reprod Immunol* 2008; 59:259–265.
22 Krigstein M, Sacks G. Prednisolone for repeated implantation failure associated with high NK cell levels: case report and risk-benefit analysis. Submitted.
23 Ogasawara M, Aoki K: Successful uterine steroid therapy in a case with a history of ten miscarriages. *Am J Reprod Immunol* 2000; 44:253–255.
24 Quenby S, Farquharson R, Young M, Vince G: Successful pregnancy outcome following 19 consecutive miscarriages: case report. *Hum Reprod* 2003; 18:2562–2564.
25 Cooper MA, Fehniger TA, Caligiuri MA: The biology of human natural killer-cell subsets. *Trends Immunol* 2001; 22:633–640.
26 Yovel G, Shakhar K, Ben-Eliyahu S: Effects of sex, menstrual cycle, and oral contraceptive use on the number and activity of natural killer cells. *Gynecol Oncol* 2001; 81:254–262.
27 Lukassen H, Joosten I, Van Crananbroek B, Van Lierop M, Bulten J, Braat D, et al.: Hormonal stimulation for IVF treatment positively affects the CD56 + bright/CD56 + Dim NK cell ratio in the endometrium during the window of implantation. *Mol Hum Reprod* 2004; 10:513–520.
28 Gleeson M, Bishop NC: The T cell and NK cell immune response to exercise. *Ann Transplant* 2005; 10:43.
29 Suzui M, Kawai T, Kimura H, Takeda K, Yagita H, Okumura K, Shek PN, Shephard RJ: Natural killer cell lytic activity and CD56 (dim) and CD56(bright) cell distributions during and after intensive training. *J Appl Physiol* 2004; 96:2167–2173.
30 Caggiula AR, McAllister CG, Matthews KA, Berga SL, Owens JF, Miller AL: Psychological stress and immunological responsiveness in normally cycling, follicular-stage women. *J Neuroimmunol* 1995; 59:103–111.
31 Moffett A, Loke C: Immunology of placentation in eutherian mammals. *Nat Rev Immunol* 2006; 6:584–594.
32 Teklenburg G, Salker M, Heijnen C, Macklon NS, Brosens JJ: The molecular basis of recurrent pregnancy loss: impaired natural embryo selection. *Mol Hum Reprod* 2010; 16:886–895.
33 Russell P, Anderson L, Lieberman D, Tremellen K, Yilmaz H, Cheerala B, Sacks G: The distribution of immune cells and macrophages in the endometrium of women with recurrent reproductive failure. *J Reprod Immunol* 2011; 91:90–102.
34 Fay L, Smith S, Chapman M, Anderson L, Russell P, Sacks G: NK cell analysis in women with recurrent reproductive failure: correlation between blood and endometrium. *ANZJOG* 2007; 47:A35–A36.
35 Park DW, Lee HJ, Park CW, Hong SR, Kwak-Kim J, Yang KM: Peripheral blood NK cells reflect changes in decidual NK cells in women with recurrent miscarriages. *Am J Reprod Immunol* 2010; 63:173–180.
36 Lachapelle MH, Miron P, Hemmings R, Roy DC: Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion. Altered profile and pregnancy outcome. *J Immunol* 1996; 156:4027–4034.

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