

Detailed analysis of peripheral blood natural killer (NK) cells in women with recurrent miscarriage

K. King¹, S. Smith^{1,2}, M. Chapman¹, and G. Sacks^{1,3}

¹St George Hospital, UNSW and IVF Australia, Sydney, Australia ²South Eastern Area Laboratory Services (SEALS) Pathology, St George Hospital, Kogarah, NSW 2217, Australia

³Correspondence address. IVF Australia, Level 16, Westfield Tower 2, 101 Grafton Street, Bondi Junction, NSW 2022, Australia. E-mail: gsacks@ivf.com.au

BACKGROUND: Increased peripheral blood natural killer (NK) cell activity has been associated with unexplained reproductive failure including recurrent (three or more) miscarriages (RM). Studies have reported abnormalities in both numbers (absolute and proportion) and activation. This study assessed numerous NK cell parameters to determine which (if any) are altered in women with RM compared with controls, which parameter best differentiated women with RM from controls, and what NK levels should be considered high.

METHODS: Luteal-phase blood samples from women with RM ($n = 104$) and controls ($n = 33$) were analysed by four-colour flow cytometry. NK cells were analysed as a percentage of lymphocytes, total NK concentration, CD56^{Dim} subtype concentration and percentage, activated CD69⁺CD56^{Dim} subtype concentration and percentage and CD56^{Bright}:CD56^{Dim} subtype ratio. Women with RM were analysed in two subgroups: those positive in ≥ 1 RM screening tests (karyotype, uterine, antiphospholipid syndrome, thrombophilia) ($n = 48$) and those who had negative screening tests ($n = 56$).

RESULTS: Women with RM had significantly higher NK percentage ($P < 0.001$), and significantly lower CD56^{Bright}:CD56^{Dim} ratio ($P < 0.05$) than controls. NK percentage was the only significantly higher variable in the RM screening test negative subgroup ($P < 0.01$). A ROC analysis (AUC = 0.71) found that an NK percentage $> 18\%$ was highly specific for women with RM (97.0%), and defined 12.5% of women with RM as having high NK percentage, compared with 2.9% of controls.

CONCLUSION: Women with RM have altered peripheral blood NK parameters. NK cells as a percentage of lymphocytes best discriminated RM and control populations. Women with RM and high NK levels may have an immunological disorder.

Key words: recurrent miscarriage / natural killer cells / early pregnancy loss / flow cytometry / immunology of pregnancy

Introduction

Recurrent miscarriage (RM), defined as three or more consecutive pregnancy losses prior to 20 weeks, occurs in 1–3% of women (Choudhury and Knapp, 2000; Carrington *et al.*, 2005). Up to half of these cases remain unexplained by routine RM screening (Druckmann and Druckmann, 2005). RM causes significant psychosocial morbidity, and for many couples the label of 'unexplained' is unacceptable.

The immune system has long been implicated in miscarriage. Medawar (1953) proposed that pregnancy involved immune modulation to protect a fetal semi-allograft from rejection, and much subsequent work defined a role for altered maternal lymphocyte profiles and trophoblast MHC expression (Sacks *et al.*, 1999). Wegmann *et al.* (1993), in a landmark paper, changed our view of maternal immunology by demonstrating that the immune system was not universally suppressed, but rather shifted to favour type 2

(antibody-mediated) over type 1 (cell-mediated) responses. It was then a simple extrapolation that an aberrant type 1 response may cause miscarriage (Raghupathy, 1997). While potential mechanisms have been described for achieving the type 2 shift for normal pregnancy involving progesterone and progesterone-induced binding factor (Roth *et al.*, 1996; Szereres-Barthos and Wegmann, 1996), and the tryptophan catabolizer IDO (Munn and Mellor, 1998), it has also been recognized that maternal immune modulation is more complex (Germain *et al.*, 2007; Chaouat, 2008). There has been renewed interest in previous observations that certain elements of the maternal innate immune system are activated in normal pregnancy (Chaouat *et al.*, 1999; Sacks *et al.*, 1999; Sacks *et al.*, 2003; Germain *et al.*, 2007). Thus, it is of interest that maternal innate natural killer (NK) cells are strikingly suppressed in normal early pregnancy (Szereres-Barthos and Wegmann, 1996; Sacks *et al.*, 2003). It is hypothesized that NK cells that are not suppressed (or are indeed

activated) could cause a type I shift and miscarriage in some women (Chaouat, 2008).

NK cells are innate lymphocytes with a $CD3^{-}CD56^{+}$ phenotype. The $CD56^{+Bright}$ NK subset is $CD16^{-}$, has a high IL-2 affinity and produce cytokines. The $CD56^{+Dim}$ subset is $CD16^{+}$, has moderate IL-2 affinity and orchestrate NK cytotoxicity (Cooper *et al.*, 2001). These subsets also express different monocyte-derived cytokine and MHC-recognizing receptors including the activating receptor CD69 (Cooper *et al.*, 2001; Coulam and Rousev, 2003). NK cells are present in the peripheral blood and in the uterine tissue, where they appear to regulate trophoblast invasion (Moffett-King, 2002; Moffett *et al.*, 2004). NK cells are the dominant uterine immune cell in pregnancy (Vince and Johnson, 2000; Koopman *et al.*, 2003), although their activity appears to be suppressed (Szereres-Barthos and Wegmann, 1996). These cells are predominantly $CD56^{+Bright}$, whereas this subset represents only 10% of the peripheral blood population, and the relationship between peripheral and uterine NK cells is unclear (Rai *et al.*, 2005).

Several studies have examined the link between altered peripheral NK levels and RM. Women with RM have been reported to have high NK cytotoxicity measured by Cr^{51} releasing assay (Aoki *et al.*, 1995; Shakhar *et al.*, 2003). Increased NK numbers were first reported by Beer *et al.* (1996) as a percentage of total peripheral lymphocytes. This study of 81 women with RM and 17 controls proposed that NK levels $>18\%$ should be considered extremely high. Little statistical detail was given to support their conclusion. Others have found an association between this parameter and primary RM only (Shakhar *et al.*, 2003). Few papers have specifically reported on the relationship between the $CD56^{+Dim}$ NK subset and RM (Beer *et al.*, 1996; Emmer *et al.*, 2000.), with no studies demonstrating a significant difference in this parameter between RM and control women. Similarly, there is little information on the relationship between the activation marker CD69 and RM. A small study ($n=40$ cases, $n=13$ controls) reported that NK cells from women with RM stimulated *in vitro* expressed more CD69 than NK cells from controls (Ntrivalas *et al.*, 2001). And a recent unconfirmed report has shown that NK cells from women with RM do appear to have increased CD69 expression (Prado-Drayer *et al.*, 2008).

Peripheral NK cell analysis is further confounded as the normal NK range for women of reproductive age is unclear. Bisset *et al.* (2004) reported a large physiological NK range for females (5.33–20.25% of total peripheral lymphocytes); however, this study did not exclude women with reproductive problems. Additionally, biopsychosocial variables may influence NK levels. Men have higher levels than women (Giglio *et al.*, 1994; Yovel *et al.*, 2001), and NK parameters can be transiently raised by acute stress and exercise (Goebel and Mills, 2000; Hosaka *et al.*, 2002; Bouillon *et al.*, 2006; Shakhar *et al.*, 2006), and women undergoing ovarian stimulation for IVF (Giuliani *et al.*, 1998). There is understandable concern that the day of the menstrual cycle may also have an effect, although data are conflicting. NK numbers have been reported to be higher in the luteal phase (Yovel *et al.*, 2001) or unchanged throughout the cycle (Giuliani *et al.* 1998), and NK cytotoxicity has been reported to be lower in the luteal phase (Souza *et al.*, 2001) or unchanged (Yovel *et al.*, 2001). In any case, this variable needs to be controlled to ensure consistency of results.

This study aimed to determine whether there was a real difference in preconceptual peripheral NK parameters between women with a

history of RM and healthy control women, to ascertain which parameters best differentiated these two cohorts, and to determine what NK levels should be considered high.

Materials and Methods

About 104 non-pregnant women with a history of three or more consecutive miscarriages (61 nulliparous and 43 parous women) were compared with 33 healthy control women (14 nulliparous and 19 parous women). For the sake of consistency and to coincide with other tests, all blood samples were taken in the mid-luteal phase. Clinical data from both cohorts were obtained retrospectively to conduct a baseline analysis for the variables of patient age, number of pregnancies, number of consecutive miscarriages, number of live births, body mass index, past medical and surgical history and medications. The RM cohort was then further subdivided into RM^{+} women who tested positive to one or more RM screening tests, and RM^{-} women who tested negative to all screening tests or who had been adequately treated for an abnormality and continued to miscarry. These tests included male and female karyotype, hormone tests (FSH, LH, testosterone, SHBG), diabetes screen (insulin, BSL, HbA1c), comprehensive thrombophilia screen (FBC, protein-S, protein-C, AT3, activated protein-C resistance, prothrombin gene mutation, factor V Leiden, anticardiolipin antibodies, lupus inhibitor, MTHFR mutation), sperm tests (DNA fragmentation) and anatomical tests (ultrasound and HyCoSy/hysterosalpingogram/hysteroscopy). It was hypothesized that couples in the RM^{-} group would be particularly frustrated with their diagnosis of unexplained RM, and might benefit from further investigation.

Prior to staining for surface marker analysis, a white cell count and lymphocyte percentage was obtained from the Sysmex XE2100 Haematology analyser (Roche Diagnostics). Surface marker analysis on peripheral blood samples was performed using direct immunofluorescence and a lyse/wash protocol and was performed within 4 h of collection. 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), 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). For NK subset determination, 2000 NK cells (CD3 negative, CD56 positive) were collected, 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 (Fig. 1).

Results were analysed with GraphPad Prism software (San Diego, CA). Mann–Whitney and Fisher's exact tests were used to compare continuous and dichotomous variables, respectively.

Results

Women in the RM cohort were aged 25–49 (mean = 36.9) and experienced on average 5.34 pregnancies (range = 5–17) and 4.4 miscarriages (range = 3–15), conceived either naturally or through IVF. Controls were aged 20–47 (mean = 34.4), had experienced two or more miscarriages (mean = 0.303), and had an average of

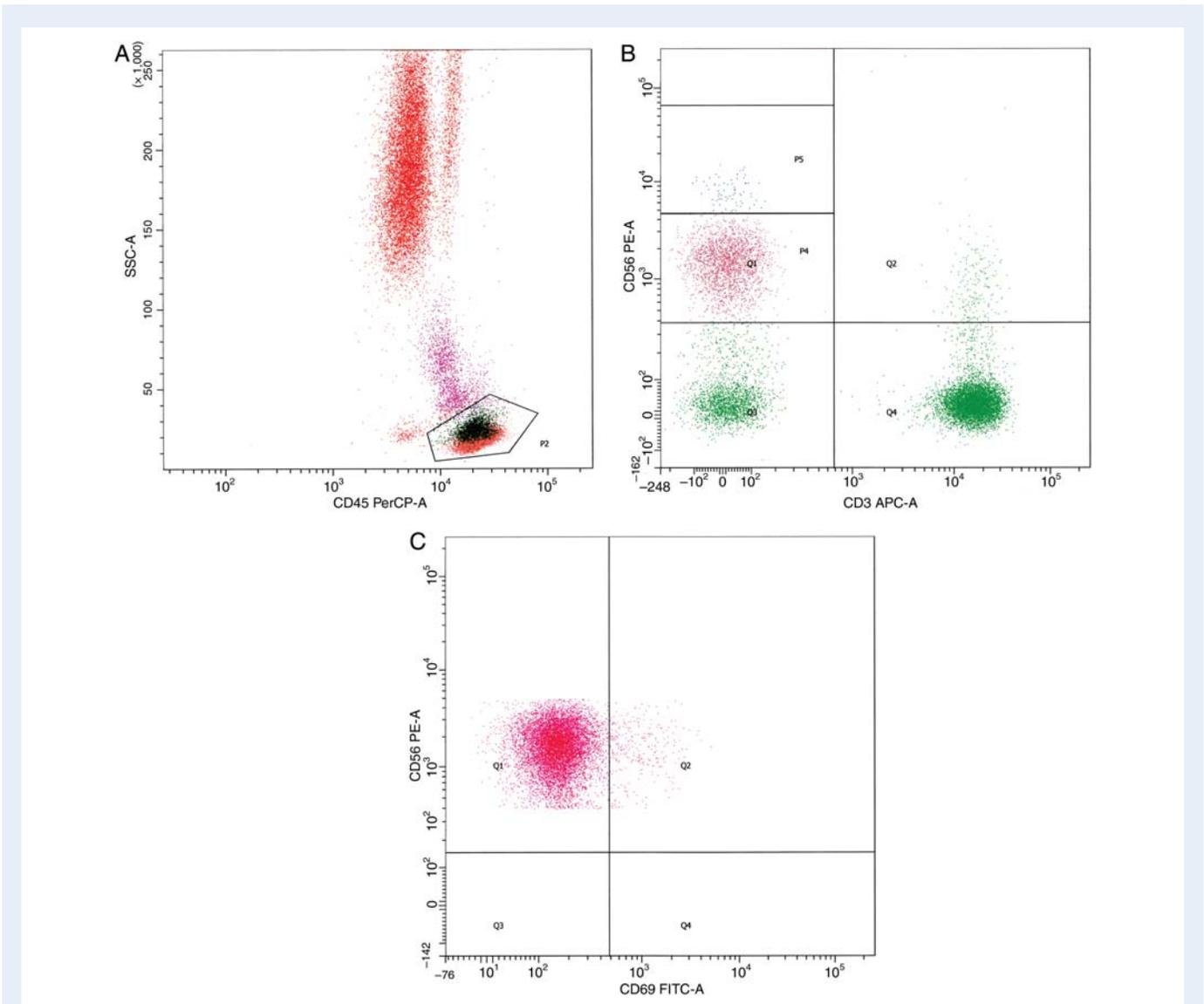


Figure 1 Identification and enumeration of NK cells and subsets by four-colour flow cytometry.

(A) CD45 is measured against SSC (side scatter)-height. P2 contains lymphocytes. (B) Cells contained within P2 are displayed on a plot of CD3 versus CD56 expression. Cells negative for CD3 and positive for CD56 are NK cells. Those with bright CD56 expression (the CD56^{+Bright} subset) lie in the region P5, and those with dim CD56 expression (the CD56^{+Dim} subset) lie within P4. (C) An example of the measurement of CD69 expression on CD56^{+Dim} NK cells. Those that lie in the upper right quadrant (Q2) are positive for CD69.

1.18 live births. There was no significant difference between RM and control cohorts for the variables of age, BMI, number of cigarettes currently smoked per day or units of alcohol consumed per week. No subject in either group took excessive alcohol, and there were few smokers in either cohort. Comparison of past medical and surgical histories revealed a higher prevalence of known autoimmune disease prior to testing in women with RM compared with controls. These included the presence of Graves' disease ($n = 3$), Hashimoto's disease ($n = 3$), SLE and antiphospholipid antibodies ($n = 4$), scleroderma ($n = 3$), psoriasis ($n = 2$) and Sjogren's syndrome ($n = 1$).

The comparison of NK parameters between women with RM and controls is shown in Table I. NK cells as a percentage of total peripheral lymphocytes was significantly elevated in the RM population ($P = 0.0004$), and the CD56^{+Bright}:CD56^{+Dim} ratio was significantly

lower ($P = 0.0365$). In the RM group, the increased mean levels of NK cell concentration, CD56^{+Dim} NK cell concentration and percentage and CD56^{+Dim}CD69⁺ NK concentration did not reach statistical significance.

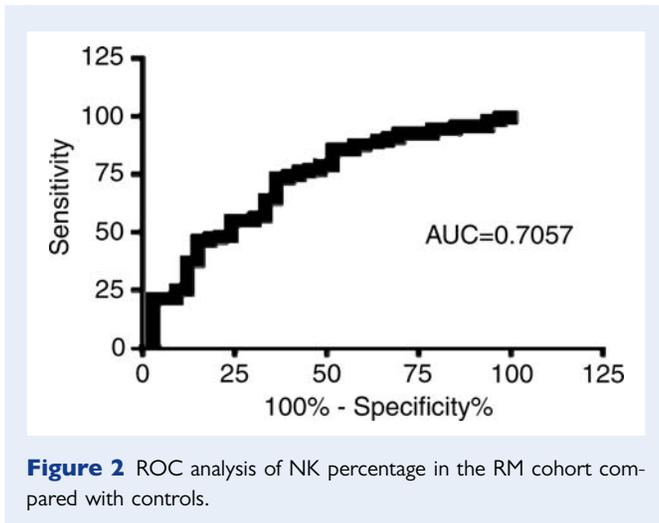
A ROC analysis (AUC = 0.706) comparing NK percentage between the RM and control cohorts was performed (Fig. 2). NK percentage >18% differentiated the cohorts with a sensitivity of 12.5 (95%CI = 6.83–20.4), specificity of 97.0 (95%CI = 84.2–99.9) and likelihood ratio of 4.12.

From the whole RM cohort, women who had had one or more previous live births were compared with women who had no live births. There was no statistically significant difference in the NK percentage between women who had had no live births (mean 12.4%) compared with those who had (10.6%, $P = 0.119$).

Table I Summary of NK parameter alterations and significance in the all RM cohort compared with controls

| Cohort | Variable | NK% | NK Conc | Dim NK% | Dim NK Conc | Bright:dim | CD69 Dim NK% | CD69 Dim NK Conc |
|-------------------------|----------|---------------|-----------|-----------|-------------|---------------|--------------|------------------|
| All RM | <i>n</i> | 104 | 104 | 104 | 104 | 104 | 90 | 90 |
| | Mean | 11.4 | 0.231 | 94.7 | 0.221 | 0.0604 | 2.77 | 0.00536 |
| | Median | 10.6 | 0.21 | 95.6 | 0.2 | 0.05 | 2.38 | 0.0043 |
| | Range | 3.08–27.5 | 0.06–0.7 | 81.2–99.1 | 0.05–0.69 | 0.01–0.22 | 0.65–38 | 0.00135–0.0154 |
| | SD | 4.87 | 0.117 | 3.85 | 11.7 | 0.461 | 3.85 | 0.00322 |
| Controls | <i>n</i> | 33 | 33 | 33 | 33 | 33 | 33 | 33 |
| | Mean | 8.80 | 0.200 | 92.5 | 0.189 | 0.0912 | 2.62 | 0.00418 |
| | Median | 5.75 | 0.17 | 94.3 | 0.16 | 0.06 | 2.29 | 0.0037 |
| | Range | 3.53–34.0 | 0.06–0.73 | 69.7–99.7 | 0.05–0.73 | 0.01–0.45 | 0.58–8.93 | 0.00097–0.0138 |
| | SD | 5.37 | 0.129 | 6.41 | 0.130 | 0.0886 | 1.67 | 0.00262 |
| <i>P</i> (Mann–Whitney) | | 0.0004 | 0.0897 | 0.0630 | 0.0584 | 0.0365 | 0.453 | 0.0527 |

Total NK percentage (NK%) = NK concentration/total peripheral lymphocytes \times 100. Total NK concentration (NK conc) = NK count \times 10^9 /l; CD56⁺Dim cell percentage (% Dim NK) = CD56⁺Dim NK concentration/NK concentration \times 100. CD56⁺Dim NK concentration (Dim NK Conc) = CD56⁺Dim NK count \times 10^9 /l. CD56⁺Bright/CD56⁺Dim ratio (Bright:dim) = CD56⁺Bright NK concentration/CD56⁺Dim concentration. Percentage of activated (CD69⁺) CD56⁺Dim NK cells (%CD69 Dim) = CD69⁺CD56⁺Dim concentration/CD56⁺Dim concentration \times 100.

**Figure 2** ROC analysis of NK percentage in the RM cohort compared with controls.

A subgroup of 46 women with RM who were screening test positive was then compared with controls (Table II). That subgroup had a significantly higher NK percentage ($P = 0.0008$), CD56⁺Dim NK percentage ($P = 0.0375$) and CD56⁺DimCD69⁺ concentration ($P = 0.0209$). CD56⁺Bright/CD56⁺Dim ratio was also significantly lower ($P = 0.0216$) and no other variables differed significantly between groups. Profiling of RM screening test results revealed a high anticardiolipin antibody (ACA) prevalence in this group (40.9%). In a subanalysis, women with ACAs were compared with women without ACAs and controls. NK percentage and concentration were significantly higher in those with ACAs compared with those without ACAs ($P = 0.0417$ and $P = 0.0477$, respectively). Women with RM and ACAs had significantly higher NK percentage and concentration, CD56⁺Dim NK percentage and concentration and CD56⁺DimCD69⁺ concentration. CD56⁺Bright/CD56⁺Dim ratio was significantly lower. NK percentage was the only variable that was significantly higher in the ACA-negative RM group compared with controls ($P = 0.002$).

The RM screening test of negative group ($n = 58$) was compared with controls (Table II). NK percentage was the only variable that was significantly increased in the RM⁻ group ($P = 0.0008$).

Discussion

This study supports previous reports that women with RM have altered peripheral blood NK parameters (increased numbers and/or levels of activation) compared with control women without a history of RM (Ntrivalas et al., 2001; Beer et al., 1996; Shakhar et al., 2003). Moreover, women in the RM⁻ cohort, who are perhaps most frustrated by having no cause found for RM, had significantly raised NK percentage. Emmer et al. (2000) failed to detect a significant difference in preconceptual NK percentage between women with RM and controls, possibly due to the broader analysis of including women with two or more miscarriages (rather than more than three in this study). Our study found that using NK percentage to define high NK levels identified women with RM with a very high specificity in this sample space, supporting Beer et al. (1996). Furthermore, the low test sensitivity supports others (Beer et al., 1996; Emmer et al., 2000; Shakhar et al., 2003) in demonstrating, reasonably, that many women with RM do not have elevated NK levels. This means that NK testing would be an ineffective way to identify women with RM from the general population, but is able to effectively identify a subpopulation of women with known RM who may benefit from immunosuppressive therapy.

Although NK percentage was the most significant difference between women with RM and controls, an overall view of all parameters analysed (including NK concentration, CD56⁺Dim concentration and percentage, CD56⁺Dim:CD56⁺Bright ratio and CD56⁺Dim expression of the activation marker CD69) was consistent with a shift in the peripheral blood NK activity in RM as has been previously reported (Beer et al., 1996; Emmer et al., 2000; Ntrivalas et al., 2001). Together, these data indicate that a peripheral shift in the NK subtypes and activation might be an indication of a genuine mechanism for immune dysfunction causing miscarriage. It is commonly argued that blood and uterine NK

Table II Summary of NK parameter alterations and significance in the RM⁺ and RM⁻ cohorts compared with controls

| Cohort | Variables | NK% | NK Conc | Dim NK% | Dim NK Conc | Bright:dim | CD69 Dim NK% | CD69 Dim NK Conc |
|---|-----------|---------------|-----------|---------------|-------------|---------------|--------------|------------------|
| Controls | <i>n</i> | 33 | 33 | 33 | 33 | 33 | 33 | 33 |
| | Mean | 8.80 | 0.200 | 92.5 | 0.189 | 0.0912 | 2.62 | 0.00418 |
| | Median | 5.75 | 0.17 | 94.3 | 0.16 | 0.06 | 2.29 | 0.0037 |
| | Range | 3.53–34.0 | 0.06–0.73 | 69.7–99.7 | 0.05–0.73 | 0.01–0.45 | 0.58–8.93 | 0.00097–0.0138 |
| | SD | 5.37 | 0.129 | 6.41 | 0.130 | 0.0886 | 1.67 | 0.00262 |
| RM ⁺ Cohort | <i>n</i> | 46 | 46 | 46 | 46 | 46 | 40 | 40 |
| | Mean | 11.9 | 0.237 | 95.2 | 0.229 | 0.544 | 2.75 | 0.00616 |
| | Median | 10.8 | 0.225 | 96.6 | 0.215 | 0.0400 | 2.47 | 0.00509 |
| | Range | 4.17–27.5 | 0.06–0.7 | 83.0–98.7 | 0.05–0.69 | 0.01–0.21 | 0.76–6.13 | 0.00135–0.0154 |
| | SD | 5.38 | 0.128 | 3.50 | 0.128 | 0.0398 | 1.18 | 0.00390 |
| <i>P</i> (Mann–Whitney) RM ⁺ versus controls | | 0.0008 | 0.0969 | 0.0375 | 0.0736 | 0.0216 | 0.311 | 0.0209 |
| RM ⁻ cohort | <i>n</i> | 58 | 58 | 58 | 58 | 58 | 50 | 50 |
| | Mean | 11.1 | 0.225 | 94.3 | 21.5 | 0.0652 | 2.78 | 0.00471 |
| | Median | 9.96 | 0.251 | 95.2 | 0.185 | 0.05 | 2.33 | 0.00397 |
| | Range | 3.08–22.0 | 0.06–0.54 | 81.2–99.1 | 0.050–0.530 | 0.01–0.22 | 0.65–8.38 | 0.00144–0.0125 |
| | SD | 4.43 | 0.108 | 4.09 | 0.1084 | 0.0595 | 1.68 | 0.00241 |
| <i>P</i> (Mann–Whitney) RM ⁻ versus controls | | 0.0025 | 0.138 | 0.188 | 0.107 | 0.129 | 0.713 | 0.225 |

Total NK percentage (NK%) = NK concentration/total peripheral lymphocytes × 100. Total NK concentration (NK conc) = NK count × 10⁹/l. CD56⁺Dim cell percentage (% Dim NK) = CD56⁺Dim NK concentration/NK concentration × 100. CD56⁺Dim NK concentration (Dim NK Conc) = CD56⁺Dim NK count × 10⁹/l. CD56⁺Bright/CD56⁺Dim ratio (Bright:dim) = CD56⁺Bright NK concentration/CD56⁺Dim concentration. Percentage of activated (CD69⁺) CD56⁺Dim NK cells(%CD69 Dim) = CD69⁺CD56⁺Dim concentration/CD56⁺Dim concentration × 100.

cells are different phenotypes, and that uterine NK cells (CD56⁺Bright) are benign, cytokine producing and probably essential for normal pregnancy (Moffett-King, 2002). However, it is now also accepted that uterine NK cells are derived (at least in part) from blood recruitment (Santoni *et al.*, 2008). Uterine NK subtype analysis is notoriously difficult to do, but one flow cytometry study has shown that women with RM have increased CD56⁺Dim subtypes (Lachapelle *et al.*, 1996). Thus it is hypothesized that excessive peripheral blood NK activity (increased numbers, increased cytotoxic CD56⁺Dim cells, increased activated cells) results in increased recruitment of CD56⁺Dim cells in the uterus. The mechanism for miscarriage is still certainly unclear, and may be the creation of a suboptimal cytokine environment rather than simply cytotoxicity, although both are possible.

The concept that peripheral NK cell over-activity is an independent marker for RM has also been suggested by the report that women with primary miscarriage have significantly increased NK percentage and concentration compared with healthy parous and nulliparous control women (Shakhar *et al.*, 2003). They showed that women with secondary miscarriage had NK percentage and concentration of an intermediate level. Our results support this study, finding increased NK percentage in women with no previous live births, compared with women with one or more previous live birth; however, this difference was not statistically significant.

Women with RM and raised ACAs had significantly higher NK percentage and concentration compared with women with RM without ACAs. This is consistent with previous reports (Beer *et al.*, 1996; Konova *et al.*, 2004). In fact we have gone further and shown numerous differences in NK parameters suggesting that women with ACAs have activated peripheral blood NK cells. It is well established that the mechanism for miscarriage in ACA positive women does not appear

to be solely thrombotic (Di Simone *et al.*, 2007). There is evidence of direct toxic effects of ACAs on trophoblast cells, and also immune dysregulation. We propose that increased NK activity in those women provides a further potential immune mechanism for poor placentation and miscarriage. Larger studies are required to assess this association, but its importance is clear from a clinical perspective. Currently women with RM and ACAs are treated with heparin and aspirin. Those with increased NK cell activity might also benefit from immune suppressive therapy (e.g. prednisone).

The use of immune suppressive therapy in women with RM has a long and controversial history. A review has concluded that there is no proven benefit in women with otherwise unexplained RM taking intravenous immunoglobulin (IVIG) or leucocyte infusions (LIT) (Porter *et al.*, 2006). However several small studies suggest that these therapies may benefit subsets of RM patients with immunological abnormalities. In particular, IVIG and LIT have been shown to reduce NK levels (Szpakowski *et al.*, 2001; Perricone *et al.*, 2006) or cytotoxicity (Kotlan *et al.*, 2006), and these effects have been associated with higher live birth rates (Check *et al.*, 1995; Kwak *et al.*, 2000; Kotlan *et al.*, 2006; Perricone *et al.*, 2006). Prednisolone is a further treatment option that can suppress NK cell activity (Thum *et al.*, 2008), and has been reported to be effective in women with RM in anecdotal reports (Quenby *et al.*, 2003). One large trial failed to demonstrate benefit in women with RM and raised autoantibodies (Laskin *et al.*, 1997), but there has not yet been a trial using prednisolone in women with high NK cell levels. It should be noted that all three potential therapies (IVIG, LIT and prednisolone) are used for their non-specific immune suppressive effects, but prednisolone has significant potential advantages. It is cheaper, easier to take, does not require blood screening and most clinicians are familiar with it. In view of potential side effects on mother and

fetus (Empson et al., 2005), immune therapy certainly requires caution. However, we believe that this study provides a firm rationale for large-scale clinical trials on the effect of immunosuppression in women with RM and high peripheral blood NK cells.

It is not known what link, if any, exists between uterine and peripheral blood NK cells. It is accepted that uterine NK cells appear to play an important role in the early implantation, but whether aberrations cause miscarriage is unknown. In any case, this study and others demonstrate an independent association between peripheral NK cells and RM. This is an important observation, as much debate previously has revolved around the significant differences between peripheral blood and uterine NK cells (Moffett et al., 2004) with the underlying assumption that NK cells themselves are hypothesized to be direct causes of miscarriage. We have presented a pilot study that demonstrated a strong correlation between blood and uterine NK cells (Fay et al., 2007), particularly when levels were high. If this finding was confirmed, it is possible to propose a mechanism whereby increased numbers, increased cytotoxic CD56⁺CD16⁺ subtypes and increased activated cells (expressing CD69) in the blood leads to increased levels of such cells recruited into the uterus every month, producing a hostile uterine environment for implantation. However, we also propose an alternative hypothesis. The immune system is complex and works as a systemic network. It is highly unlikely that a single cell type is the sole cause of miscarriage in those women. Raised NK cell activity has not been recognized in any other disease state that we are aware of, apart from abdominal aortic aneurysm (Forester et al., 2006). Thus perhaps we should interpret NK activity as just one measure of overall immune function. It has been proposed that, more accurately, there may be a syndrome in which the presence of various immune factors (NK cells in blood, NK cells in uterus, ACAs, thyroid antibodies, etc.) increase the likelihood of an immune reproductive disorder (Gleicher, 2002).

In conclusion, this study is one of the largest and most detailed flow cytometric analyses of preconceptual peripheral blood NK cells in women with RM. It clearly demonstrated that women with RM have significantly increased NK activity, with NK percentage being the parameter that best differentiated test and control groups. By a simple blood test, 12.5% of women with RM were found to have an NK percentage > 18% compared with only 3% of the control population. It is not yet proven that high NK levels signal a pathological mechanism predicting miscarriage. Nor is it known how NK levels come to be raised, how long they remain high, or what long-term health consequences might be. Nevertheless, we believe that randomized controlled studies are indicated to assess whether women with such high NK levels would benefit from immune therapy.

Funding

We would like to thank SEALS Pathology for providing flow cytometry services.

References

- Aoki K, Kajjura S, Matsumoto Y, Ogasawara M, Okada S, Yagami Y, Gleicher N. Preconceptional natural-killer-cell activity as a predictor of miscarriage. *Lancet* 1995;**345**:1340–1342.
- 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 fertilisation cycles. *Am J Reprod Immunol* 1996;**35**:376–382.
- Bisset LR, Lung T, Kaelin M, Ludwig E, Dubd R. Reference values for peripheral blood lymphocyte phenotypes applicable to the healthy adult population in Switzerland. *Eur J Haematol* 2004;**72**:203–212.
- Bouillon LE, Flynn M, Lambert C, Fahlman M, Braun W, Choi D. Exercise during late-follicular menstrual phase: influence on immune parameters. *J Sports Med Phys Fitness* 2006;**46**:143–154.
- Carrington B, Sacks G, Regan L. Recurrent miscarriage: pathophysiology and outcome. *Curr Opin Obstet Gynecol* 2005;**17**:591–597.
- Chaouat G. Current knowledge on natural killer cells, pregnancy and pre-eclampsia. Introduction. *Reprod Biomed Online* 2008;**16**:170–172.
- Chaouat G, Cayol V, Mairovix V, Dubanchet S. Localisation of the TH2 cytokines IL-3, IL-4, IL-10 at the feto-maternal interface during human and murine pregnancy and lack of requirement for Fas/Fas ligand interaction for a successful allogenic pregnancy. *Am J Reprod Immunol* 1999;**42**:1–13.
- Check JH, Tarquini P, Gandy P, Lauer S. A randomised study comparing the efficacy of reducing the spontaneous abortion rate following lymphocyte immunotherapy and progesterone treatment versus progesterone alone in primary habitual aborters. *Gynaecol Obstet Invest* 1995;**39**:257–261.
- Choudhury SR, Knapp L. Human reproductive failure I: Immunological factors. *Hum Reprod Update* 2000;**7**:113–134.
- Cooper MA, Fehniger T, Caligiuri M. The biology of human natural killer cell subsets. *Trends Immunol* 2001;**22**:633–640.
- Coulam CB, Roussev RG. Correlation of NK cell activation and inhibition markers with NK cytotoxicity among women experiencing immunologic implantation failure after in vitro fertilization and embryo transfer. *J Assist Reprod Genet.* 2003;**20**:58–62.
- Di Simone N, Luigi MP, Marco D, Fiorella DN, Silvia D, Clara DM, Alessandro C. Pregnancies complicated with antiphospholipid syndrome: the pathogenic mechanism of antiphospholipid antibodies: a review of the literature. *Ann N Y Acad Sci* 2007;**1108**:505–514.
- Druckmann R, Druckmann M. Progesterone and the immunology of pregnancy. *J Steroid Biochem Mol Biol* 2005;**97**:389–396.
- Emmer PM, Nelen W, Steegers E, Hendricks J, Veerhoek M, Joosten I. Peripheral natural killer cytotoxicity and CD56posCD16pos cells increase during early pregnancy in women with a history of recurrent spontaneous abortion. *Hum Reprod* 2000;**15**:1163–1169.
- Empson M, Lassere M, Craig J, Scott J. Prevention of recurrent miscarriage for women with antiphospholipid antibody or lupus anticoagulant. *Cochrane Database Syst Rev* 2005;**18**:CD002859.
- Fay L, Smith S, Russell P, Chapman M, Sacks G. *NK cell Analysis in Women with Recurrent Reproductive Failure: There is a Clinical Correlation between Peripheral Blood and Endometrial Biopsy Samples*. Hobart: Oral presentation, Fertility Society of Australia, 2007.
- Forester ND, Cruickshank SM, Scott DJ, Carding SR. Increased natural killer cell activity in patients with abdominal aortic aneurysm. *Br J Surg* 2006;**93**:46–54.
- Germain SJ, Sacks GP, Sooranna SR, Sargent IL, Redman CW. Systemic inflammatory priming in normal pregnancy and preeclampsia: the role of circulating syncytiotrophoblast microparticles. *J Immunol* 2007;**178**:5949–5956.
- Giglio T, Imro M, Filaci G, Scruelletti M, Puppo F, De Cecco L, Indiveri F, Costantini S. Immune cell circulating subsets are effected by gonadal functioning. *Life Sci* 1994;**54**:1305–1312.
- Giuliani A, Schoell W, Auner J, Urdl W. Controlled ovarian hyperstimulation in assisted reproduction: effect on the immune system. *Fertil Steril* 1998;**70**:831–835.

- Gleicher N, Vidali A, Karande V. The immunological "Wars of the Roses": disagreements amongst reproductive immunologists. *Hum Reprod* 2002; **17**:539–542.
- Goebel M, Mills P. Acute physiological stress and exercise changes in the peripheral leukocyte adhesion molecule expression and density. *Psychosom Med* 2000; **62**:664–670.
- Hosaka T, Matsubayashi H, Sugiyama Y, Izumi S, Makino T. Effect of psychiatric intervention on natural killer cell activity and pregnancy rate. *Gen Hosp Psychiatry* 2002; **24**:353–356.
- Konova E, Ivanova I, Petrova P, Popov I, Andreeva K, Lukanov T, Gecheva S. Effect of intravenous gamma-globulin therapy on lymphocyte population in pregnant women with antiphospholipid antibodies. *Akusherstvo i Ginekologija* 2004; **43**:3–10.
- Koopman L, Kopcow H, Rybalov M, Bowson J, Organe J, Schatz F, Masch R, Lockwood CJ, Schachter AD, Park PJ et al. Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *J Exp Med* 2003; **198**:1201–1212.
- Kotlan B, Padanyi A, Batorfi J, Fulop V, Szigetvari I, Rajczy K, Penzes M, Gyodi E, Reti M, Petranyi G. Alloimmune and autoimmune background in recurrent pregnancy loss—successful immunotherapy by intravenous immunoglobulin. *Am J Reprod Immunol* 2006; **55**:331–340.
- Kwak JY, Kwak FM, Gilman-Sachs A, Beaman KD, Cho DD, Beer AE. Immunoglobulin G infusion treatment for women with recurrent spontaneous abortions and elevated CD56+ natural killer cells. *Early Pregnancy* 2000; **4**:154–164.
- 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.
- Laskin CA, Bombardier C, Hannah ME, Mandel FP, Knox Ritchie JW, Farewell V, Farine D, Spitzer K, Fielding L, Soloninka CA et al. Prednisone and aspirin in women with autoantibodies and unexplained recurrent fetal loss. *N Engl J Med* 1997; **337**:148–153.
- Medawar PD. Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. *Symposium of the Society for Experimental Biology* 1953:320.
- Moffett A, Regan L, Braude P. Natural killer cells, miscarriage and infertility. *BMJ* 2004; **329**:1283–1285.
- Moffett-King A. Natural killer cells and pregnancy. *Nat Rev Immunol* 2002; **2**:656–663.
- Munn D, Mellor A. Prevention of allogenic fetal rejection by tryptophan catabolism. *Science* 1998; **281**:1191–1194.
- Ntrivalas E, Kwak-Kim J, Gilman-Sachs A, Chung-Bang H, Ng S, Beaman K, Mantouvalos HP, Beer AE. Status of peripheral blood natural killer cells in women with recurrent spontaneous abortions and infertility of unknown aetiology. *Hum Reprod* 2001; **16**:855–861.
- Periconne R, Di Muzio G, Perricone C, Giacomelli R, De Nardo D, Fontana L, De Carolis C. High levels of peripheral blood NK cells in women suffering from recurrent spontaneous abortion are reverted form high dose intravenous immunoglobulins. *Am J Reprod Immunol* 2006; **55**:232–239.
- Porter TF, LaCoursiere Y, Scott JR. Immunotherapy for recurrent miscarriage. (Review). *Cochrane Database of Syst Rev* 2006; Art. No.: CD000112. DOI: 10.1002/14651858.CD000112.pub2.
- Prado-Drayer A, Teppa J, Sánchez P, Camejo MI. Immunophenotype of peripheral T lymphocytes, NK cells and expression of CD69 activation marker in patients with recurrent spontaneous abortion, during the mid-luteal phase. *Am J Reprod Immunol* 2008; **60**:66–74.
- Quenby S, Farquharson R, Young M, Vince G. Successful pregnancy outcome following 19 consecutive miscarriages: case report. *Hum Reprod* 2003; **18**:2562–2564.
- Rai R, Sacks G, Trew G. Natural killer cells and reproductive failure—theory, practice and prejudice. *Hum Reprod* 2005; **20**:1123–1126.
- Raghupathy R. Th-1 type immunity is incompatible with successful pregnancy. *Immunol Today* 1997; **18**:478–482.
- Roth I, Corry D, Locksley R, Abrams J, Litton M, Fisher S. Human placental cytotrophoblast produce the immunosuppressive cytokine interleukin-10. *J Exp Med* 1996; **184**:539–548.
- Sacks G, Sargent I, Redman C. An innate view of human pregnancy. *Immunol Today* 1999; **20**:114–118.
- Sacks GP, Redman CW, Sargent IL. Monocytes are primed to produce the Th1 type cytokine IL-12 in normal human pregnancy: an intracellular flow cytometric analysis of peripheral blood mononuclear cells. *Clin Exp Immunol* 2003; **131**:490–497.
- Santoni A, Carlino C, Stabile H, Gismondi A. Mechanisms underlying recruitment and accumulation of decidual NK cells in uterus during pregnancy. *Am J Reprod Immunol* 2008; **59**:417–424.
- Shakhar K, Shamgar B, Loewenthal R, Rosenne E, Carp H. Differences in number and activity of peripheral natural killer cells in primary versus secondary recurrent miscarriage. *Fertil Steril* 2003; **80**:368–375.
- Shakhar K, Rosenne E, Loewenthal S, Shakhar G, Carp H, Ben-Eliyahu S. High NK cell activity in recurrent miscarriage: What are we really measuring? *Hum Reprod* 2006; **21**:2421–2425.
- Souza S, Castro F, Mendonça H, Palma P, Morais F, Ferriani R, Voltarelli JC. Influence of menstrual cycle on NK activity. *J Reprod Immunol* 2006; **50**:151–159.
- Szereres-Barthos J, Wegmann T. A progesterone-dependent immunomodulatory protein alters the Th1/Th2 balance. *J Reprod Immunol* 1996; **31**:81–95.
- Szapkowski A, Malinowski A, Zeman K, Wilczynski J, Kolasa D, Nowak M, Wladzinski J, Kaminski T, Szapkowski M. The influence of paternal lymphocytes immunization on percentage of peripheral blood CD16+CD56+ cells in women with recurrent spontaneous abortion. *Gynecol Pol* 2001; **17**:1063–1068.
- Thum MY, Bhaskaran S, Abdalla HI, 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.
- Vince G, Johnson P. Leukocyte populations and cytokine regulation in human uteroplacental tissues. *Biochem Soc Trans* 2000; **28**:191–195.
- Wegmann T, Lin H, Guilbert L, Mosmann T. Bidirectional cytokine interactions in the maternal-fetal relationship: Is successful pregnancy a Th2 phenomenon? *Immunol Today* 1993; **14**:353–357.
- 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.

Submitted on June 30, 2009; resubmitted on September 1, 2009; accepted on September 7, 2009