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

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Introduction

- Recurrent miscarriage
  - ☺ ≥3 consecutive pregnancy losses , before GA 20wks
  - ☺ Prevalence: 1-3%
  - ☺ > 50% → unexplained
  - ☺ Cause significant psychosocial morbidity

Introduction

- Immune system in pregnant women
  - Involved immune modulation to protect a fetal semi-allograft from rejection. (Medawar, 1953)
  - Role of maternal lymphocyte profiles and trophoblast MHC expression. (Sacks el at., 1999)
  - The immune system was not universally suppressed , but rather shifted to favour type 2 (Ab-mediated) over type 1 (cell-mediated) responses . (Wegmann et al., 1993)
  - An aberrant type 1 response may cause miscarriage.(Raghupathy, 1997)
  - Type 2 shift for normal pregnancy involving P and P-induced binding factor and tryptophan catabolizer IDO.(Roth et al., 1996)

Introduction

- Interest one of the certain elements in maternal innate immune system → NK cells
  - ☺ NK cells are strikingly suppressed in normal early pregnancy. (Szereres-Barthos and Wegmann, 1996)
  - ☺ NK cell are not suppressed (or are indeed activated) could cause a type 1 shift and miscarriage in some women . (Chaouat, 2008)

NK cells

- Innate lymphocytes , with $CD3^{-}CD56^{+}$
- $CD56^{+Bright}$ NK subset : CD16-, high IL-2 affinity, produce cytokines
- $CD56^{+Dim}$ NK subset: CA16+, moderate IL-2 affinity , orchestrate NK cytotoxicity
- CD69 : MHC- Recognizing , activating receptors
- Present in the peripheral blood and uterine tissue →regular trophoblast invasion (Moffett-King,2002)
- Is dominant uterine immune cell in pregnancy (Vince and Johnson, 2000)
- $CD56^{+Bright}$ predominant in uterus , only 10% of peripheral blood population.

Relationship between NK and RM

- Women with RM have high NK cytotoxicity (Aoki, 1995; Shakhar, 2003)
- NK levels >18% should be considered extremely high . (Beer et al.,1996)
- Relationship between the CD56+Dim NK subset and RM →few papers reported (Beer , 1996; Emmer, 2000)
- Difference in NK levels between RM and control women → no studies
- Relationship between CD 69 and RM
  - ☺ NK cells from women with RM stimulated in vitro expressed more CD69 than NK cells from controls . (Ntrivalas et al., 2001)
  - ☺ Women with RM appear to have increased CD69 expression . (Prado-Drayer et al., 2008)

Peripheral NK cell analysis

- Normal NK range for women of reproductive age→ unclear
- A large NK range for female : 5.33-20.25% (Bisset , 2004)
- Biopsychosocial variables may influence NK levels (higher)
  - ☺ Men
  - ☺ Acute stress
  - ☺ Exercise
  - ☺ ovarian stimulation for IVF
  - ☺ Menstrual cycle (luteal phase)

Aim of this study

- Determine whether there was a real difference in preconceptual peripheral NK parameters between women with RM and healthy control women
- Ascertain which parameters best differentiated these 2 cohorts
- Determine what NK levels should be considered high

Materials and Methods

- 104 non-pregnant women with RM (61 nulliparous , 43 parous)
- 33 healthy control women (14 nulliparous, 19 parous)
- Blood samples in the mid-luteal phase
- Baseline analysis
  - Age
  - No. Of pregnancies
  - No. Of consecutive miscarriage
  - No. Of live births
  - BMI
  - Past medical and surgical hx and medications

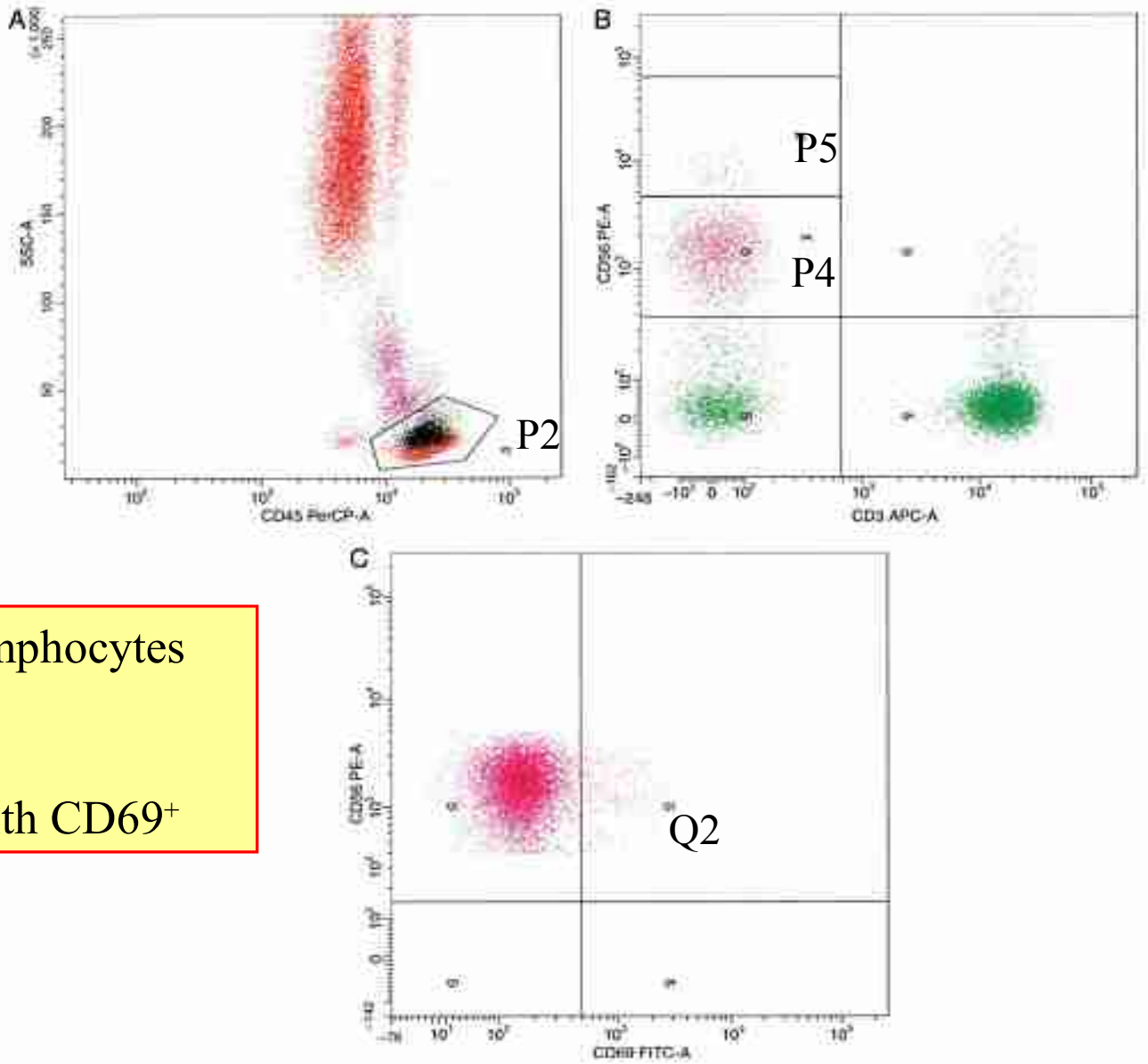
- RM+ : positive to ≥1 RM screening tests
- RM- : negative to all screening tests or had been treated for an abnormality and continued to miscarry .

- RM screening test
  - Male and female karyotype
  - Hormone test(FSH, LH,T,SHBG)
  - Diabetes screen (insulin, BSL, HbA1c)
  - Thrombophilia screen
  - Sperm test (DNA fragmentation)
  - Anatomical test(sono and HyCoSy/HSG/ HSC)

- % of white cell count and lymphocyte %
- Surface marker analysis (use peripheral blood)
  - CD45-PerCP
  - CD3-APC
  - CD19-APC
  - CD56-PE
  - CD69-FITC
  - CD14-PE
  - CD16-FITC

- NK subset determination (use flow cytometry)
  - Collected 2000NK cells (CD3-, CD56+)
  - 10000NK cells for measurement of activation using CD69
  - total NK cells , $CD56^{+Dim}$ subset ,activated $CD69^{+}$ $CD56^{+Dim}$ subset→expressed as % and absolute count

- Result analysis
  - GraphPad Prism software
  - Mann-Whitney → continuous variables
  - Fisher's exact test → Dichotomous variables



- P2 : contains lymphocytes
- P5 : CD56$^{+Bright}$
- P4: CD56^{+Dim}
- Q2: CD56^{+Dim} with CD69^{+}

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.

Results

Results of baseline analysis

	Control	RM
Age	20-47 (mean=34.4)	25-49(mean=36.9)
No.of pregnancies	—	5.34 (range= 5-17)
Miscarriages	≥2 miscarriage →mean=0.303	4.4 (range= 3-15)
Live births	1.18	—

- No significant difference between RM and control
  - ☺ age; BMI; no.of cigarettes /day ; units of alcohol consumed /wk
- Higher prevalence of autoimmune disease in RM cohort
  - ☺ Grave's disease (3) ; Hashimoto's disease (3) ; SLE + APA (4) ; scleroderma (3) ; psoriasis (2); Sjogren's syndrome (1) .

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	*n*	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	*n*	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
P (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 × 100. Total NK concentration (NK conc) = NK count × 10^9/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 × 10^9/l. $CD56^{+Bright}/CD56^{+Dim}$ ratio (Bright:dim) = $CD56^{+Bright}$ NK concentration/$CD56^{+Dim}$ concentration. Percentage of activated ($CD60^+$) $CD56^{+Dim}$ NK cells (%CD69 Dim) = $CD69^+CD56^{+Dim}$ concentration/$CD56^{+Dim}$ concentration × 100.

- NK% was significantly elevated in the RM cohort
- Bright:dim ratio was significantly lower
- Other variables → not reach statistical significance.

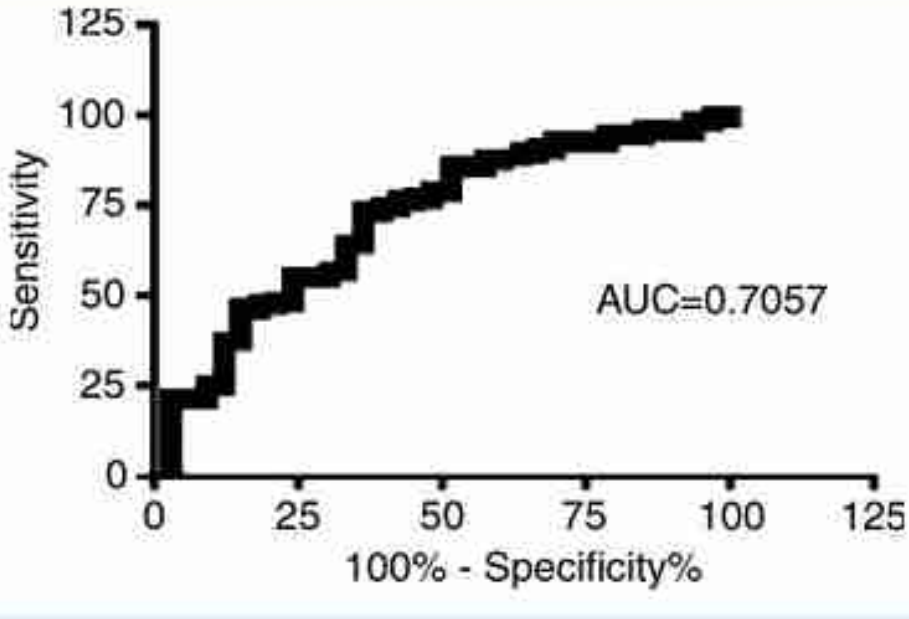


Figure 2 ROC analysis of NK percentage in the RM cohort compared with controls.

- NK% >18% differentiated the cohorts with a sensitivity of 12.5%, specificity of 97.0% and likelihood ratio of 4.12
- Women without live births vs with live birth
  - ☺ NK% → no significant difference

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

Cohort	Variables	NK% H	NK Conc	Dim NK% H	Dim NK Conc	Bright:dim L	CD69 Dim NK%	CD69 Dim NK Conc H
Controls	*n*	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	*n*	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
P (Mann–Whitney) RM^- versus controls		**0.0008**	0.0969	**0.0375**	0.0736	**0.0216**	0.311	**0.0209**

- In RM cohort , prevalence of anticardiolipn antibody : 40.9%
- RM(+) with ACAs vs control
  - ☺ significant higher → NK%, NK conc , Dim NK%, DimNK conc.
  - ☺ Significant lower → Bright : Dim ratio
- RM(+) without ACAs vs control
  - ☺ Only NK% was significant higher

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Cohort	Variables	NK% H	NK Conc	Dim NK%	Dim NK Conc	Bright:dim	CD69 Dim NK%	CD69 Dim NK Conc
Controls	*n*	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	*n*	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
P (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 × 10^9/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 × 10^9/l. $CD56^{+Bright}/CD56^{+Dim}$ ratio (Bright:dim) = $CD56^{+Bright}$ NK concentration/$CD56^{+Dim}$ concentration. Percentage of activated ($CD60^+$) $CD56^{+Dim}$ NK cells(%CD69 Dim) = $CD69^+CD56^{+Dim}$ concentration/$CD56^{+Dim}$ concentration × 100.

- Only NK % was significantly increased in the RM^- group

Discussion

- Women with RM have alter peripheral blood NK parameters (increased no. and/or levels of activation) → support previous reports
- Women in RM^- cohort , had no cause found in RM but significantly raised NK% .
- Using NK% to define high NK level
  - ☺ High specificity(97%) but low sensitivity(12.5%) in women with RM
  - ☺ NK testing would be an ineffective way to identify women with RM from the general population
  - ☺ But effectively identify a subpopulation of women with known RM who may benefit from immunosuppressive therapy .

- All parameters analysed was consistent with a shift in the peripheral blood NK activity in RM .
- Peripheral shift in the NK subtypes and activation might be an indication of a genuine mechansim for immune dysfunction causing miscarriage .
  - ☺ Blood NK vs uterine NK
    - ut NK partly derived from blood recruitment .
    - Ut NK $CD56^{+bright}$→benign, produce cytokine , essential for normal pregnancy ;
    - Ut NK $CD56^{+Dim}$ →cytotoxic , increased in RM
    - Hypothesis : peripheral blood NK activity↑→ $CD56^{+Dim}$↑→ recruitment of $CD56^{+Dim}$ in the uterus ↑
  - ☺ Mechansim for miscarriage is still unclear !!

Peripheral NK cell over-activity

- An independent marker for RM
- Shakhar et al.,2003
  - Primary miscarriage have significant increased NK% and conc .
  - Secondary miscarriage had NK% and conc of an intermiediate level .
- Our study
  - Increased NK% in women with no previous live birth→ but not statistically significant .

RM with ACAs (+)

- Had significant higher NK% and conc in this study
- Consistent with previous reports (Beer 1996; Konova, 2004)
- Mechanism for miscarriage in ACA(+)
  - Not solely thrombotic
  - Direct toxic effect on trophoblast and immue dysregulation
- Higher NK% →further potential immune mechanism for poor placentation and miscarriage
- Current treatment
  - Heparin + aspirin
  - If NK activity increased →might benefit from immunosupressive therapy (e.g. Prednisone)

Immune suppressive therapy in RM

- Still controversial
- There is no proven benefit for unexplained RM with taking IVIG or leucocyte infusion (LIT) . (Porter. 2006)
- Several small studies : these therapies may benefit subsets of RM pts with immunological abnormalities.
- IVIG and LIT → reduce NK levels or cytotoxicity, and with higher live birth rates
- Prednisolone
  - ☺ Can suppress NK cell acitivity (Thum , 2008)
  - ☺ Effective in women with RM (Quenby,2003)
  - ☺ Cheaper , easier to take , not require blood screening
- Be caution the side effects on mother and fetus .

- It is not known what link exists between uterine and peripheral blood NK cells.
- But uterine NK play an important role in the early implantation.
- A pilot study (Fay et al., 2007)
  - ☺ A strong correlation between blood and ut NK cells , particularly when levels were high .
- A possible mechanism : ↑number,↑ cytotoxic $CD56^{+\,Dim}$ subtypes and ↑activated cell (CD69+) in the blood →↑such cells recruitment into the uterus →a hostile ut enviroment for implantation

- An alternative hypothesis
  - ☺ Immune system is complex and works as a network → It's unlikely that a single cell type is the sole cause of miscarriage in RM women
  - ☺ NK activity is just one measure of overall immune function
  - ☺ May be a syndrome with various immune factors (NK cell in blood and uterus, ACAs, thryoid Ab, etc) increase the likelihood of an immune reproductive disorder

Conclusion

- This study is one of the largest and most detailed flow cytometric analyses of preconceptual peripheral blood NK cells in women with RM.
- Women with RM have significantly increased NK activity.
- NK% is 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% > 18% compared with only 3% of the control population .

Conclusion

- It is not yet proven that high NK levels signal a pathological mechansim 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 .
- We believe that randomized controlled studies are indicated to assess whether women with such high NK levels would benefit from immune therapy .