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ORIGINAL ARTICLE

Moving from peripheral blood to local uterine immunophenotype analysis in patients with poor reproductive history: pilot study of a novel technique

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Abstract

Background A complete reproductive immunophenotype is poorly described, with most focus on peripheral blood natural killer cells rather than uterine populations. There is debate regarding normal endometrial levels, with no consensus, and much controversy on correlation with implantation/miscarriage.

Aims Development and validation of a rapid endometrial assessment flow cytometry (FCM) technique, allowing determination of local lymphocyte subset ranges, comparison to peripheral blood, and patient subgroup analysis.

Methods Prospective pilot, assessing patients with prior implantation, failure offered endometrial biopsy before subsequent ART cycle, functioning as therapeutic scratch. HRT regime administered to standardise environment, and progesterone-primed mid-luteal biopsy (five completed days progestogen, P+5) analysed using comprehensive flow panel to identify lymphocyte subsets.

Results Two hundred patients were recruited in a tertiary university-affiliated ART centre. FCM identified differing lymphocyte ranges between peripheral blood and biopsy. Uterine/decidual natural killer cells are the dominant endometrial subtype. Patients with repeated implantation failure had higher uNK levels (52.4 vs 43.7%, $p = 0.01$). Conversely, B lymphocytes (0.87 vs 0.72%, $p = 0.032$), pNK (1.21 vs 0.8%, $p = 0.041$), and NK-T (2.68 vs 2.26, $p = 0.031$) cells were higher in recurrent pregnancy loss.

Conclusion FCM is widely used to assess cellular populations, but not typically employed for endometrial evaluation. FCM provides a rapid, detailed, and quantitative analysis and reduces inter-observer subjectivity bias. Detailed understanding of the normal endometrial immunophenotype, and associated deviations, may provide insight into the aetiology of infertile patients labelled "unexplained". Failure despite transfer of high grade, or proven euploid blastocysts, is a difficult problem, and endometrial profiling may help identify research areas to determine potential future therapeutic interventions for this difficult to treat population.

Keywords ART · Endometrium · Immunophenotype · Lymphocytes · Natural killer cells

Introduction

The human endometrium undergoes a remarkable series of changes during the menstrual cycle in preparation for embryonic implantation, and the local immune system plays a key role in this. The structure of the endometrium comprises a combination of cells and molecules contributing to processes of cell adhesion and signalling, all of which are active at varying stages [1]. Compared to peripheral blood, a complete uterine immunophenotype has not been well described, with no accepted technique to assess the constituent cellular populations [2]. There is even less consensus on the normal ranges for endometrial lymphocyte subsets, and whether their excess or deficiency has any clinical consequences. Flow cytometry is powerful in the assessment of immune cell profiles; however, it has been mostly employed on blood samples, not for direct endometrial analysis. Blood lymphocyte values may be easily influenced by external factors, and have no proven correlation with reproductive outcome, so have not obtained much credibility in the scientific community as a prognostic marker for reproductive medicine [3]. Failure to achieve an ongoing pregnancy using assisted reproductive technologies

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(ART) with embryos of high morphological quality is unfortunately common, and the underlying factor for the majority of these unsuccessful treatments is frustratingly categorised as "unexplained". The most common reasons in this situation are embryo factors, such as aneuploidy [4]. Most focus on treatment or testing is naturally directed towards this, ignoring the local endometrial environment, which may actually have a key role to play. There are, in fact, many non-embryological reasons for ART failure, including anatomical, infective [5], altered microbiome [6], coagulation disorders [7, 8], abnormal receptivity [9, 10], and immune-mediated theories [11, 12]. Preimplantation genetic screening (PGT) techniques allow for comprehensive embryo chromosomal analysis to be incorporated into an ART cycle with a view to improving live birth rates per embryo transfer, by excluding aneuploid blastocysts. This approach can be successful in certain patient populations, but interestingly, PGS has repeatedly shown that chromosomally normal embryos do not always lead to a baby. At least 30% of euploid screened embryos still fail to implant or lead to a miscarriage [13]. In these cases, it may be plausible to hypothesise that a uterine factor could be present, especially in younger patients. More work is needed to determine the underlying reasons for this and allow the design of studies to assess if adjunctive treatments could have a beneficial role, or not, in carefully selected patients. There is still much debate about when and how to assess the endometrium itself, either for receptivity, hostility/immunology, or both. The increasing use of comprehensive embryo chromosomal screening pretransfer could increase in interest in the uterine environment following unsuccessful treatments, particularly the fields of receptivity and reproductive immunology. There are currently no conclusively accepted criteria as to when, or even if, these tests should be considered for regular use, or how to interpret their results. A more detailed understanding of the endometrium is needed to help answer these questions.

Methodology

Peripheral blood immunophenotypes have been well described and studied, but their correlation with the endometrial environment cannot be assumed, and they have not yet been successfully linked with clinical outcomes in a fertility setting. Specialised reproductive immunophenotypes on blood samples have long been available, but no significant data has been produced to support their routine use, leading to interest in the luteal endometrium. To assess if a localised uterine assessment performs better than peripheral blood analysis, a comprehensive flow cytometry panel was designed to evaluate the endometrial lymphocyte immunophenotype in detail, utilising CD45, CD3, CD5, CD16, CD19, CD56, CD4, and CD8. This allowed detection and analysis of various key lymphocyte subgroups, including local uterine/decidual type natural killer cells (uNK; CD16−, CD56 ^{bright}), peripheral type natural killer cells (pNK: CD16+, CD56^{dim}), natural killer T cells (NK-T; CD3+, CD16−, CD56^{dim}), and B cells (CD19+). T lymphocytes were identified using CD45 and CD3, then separated into CD4+ and CD8+ cells.

A sample size of 200 patients was chosen for this pilot study to identify population averages, ranges, and centiles for the each of the parameters to be evaluated. Cases were recruited from the cohort of patients with a previous failed blastocyst transfer electing to have an endometrial scratch performed in the mid-luteal phase of the menstrual cycle immediately preceding their subsequent ART attempt, with a view to improving implantation potential. The iatrogenic injury was performed as an outpatient procedure using a Pipelle™ biopsy catheter, and rather than discarding the collected tissue as per standard practice at scratch, it was retained in RPMI media for subsequent analysis. Prior written informed consent was obtained from patients for the collecting, testing, and analysis of this tissue, and advanced approval was obtained for the pilot study to validate the biopsy technique. Residual endometrium was discarded after analysis. To maintain consistency between endometrial environment across all samples, a standardised HRT protocol using oral oestradiol hemihydrate (Estrofem 6 mg) and vaginal progesterone gel (Crinone 8% PV OD) was used for preparation, with scratch/biopsy at P+5 (cerca cycle day 21) to ensure the tissues were representative of the local environment during the anticipated window of implantation. Samples were collected over an 18-month period in a tertiary level university-affiliated ART centre. Extracted endometrium tissue was then weighed to allow the samples to be standardised, mechanically dissociated using MACs™, then evaluated by flow cytometry for co-localisation of antibodies with a staining buffer (Navios ™, Beckman Coulter UK LTD). For subgroup analysis, patients from the overall cohort with strict repeated implantation failure (RIF) and recurrent pregnancy loss (RPL) were identified. Inclusion criteria for RIF was defined as >2 unsuccessful blastocyst embryo transfers, and RPL as >2 clinically detectable miscarriages. IBM SPSS v24 was employed to analyse the output data and determine distribution, means, medians, and centiles for the key markers to provide a more complete understanding of the endometrial immunophenotpye and potential variations. Mann-Whitney *U* test was employed to compare differences between the RPL and RIF subgroups.

Results

The initial endometrial immunophenotype protocol was hypothesised and developed between late 2012 and mid-2013 following prior work on peripheral and menstrual blood in patients with recurrent miscarriage and implantation failure. Cases were recruited for the pilot study from May 2013.

Table 1 Age and obstetric history of the various patient subgroups investigated RPL, recurrent pregnancy loss. *RIF*, repeated implantation failure

	RPL	RIF	Other	All
n	62	64	74	200
Age	38.0	37.9	37.5	37.8
Live births	21	0	29	50
Miscarriages	170	0	39	209

Peripheral blood had insufficient data supporting its use in this setting, and menstrual blood analysis was limited by difficulties with specimen collection, potential contamination, and the fact that the wrong phase of the cycle was being assessed, hence endometrial assessment was the logical transition. Two hundred patients choosing to have an elective endometrial scratch as part of their ART cycle agreed to have the collected tissue tested for further analysis as part of the study protocol (Table 1), with samples collected over the subsequent 18 months. The tissue preparation and analysis techniques proved successful, and the target endometrial lymphocyte population was shown to successfully survive the tissue procurement, and preparation steps, being clearly detectible on flow cytometric analysis (Fig. 1). The lymphocyte subtypes of interest were then easily identified in the endometrium by CD45-mediated cluster differentiation marker FCM (Fig. 2). Immunophenotype results were compared to peripheral blood immunophenotypes analysed over the study period, and marked differences were noted in all parameters tested, between these differing immunological environments (Table 2). In the endometrium, natural killer cells predominate, and constitute almost 5 times their corresponding component in peripheral blood (48.5% vs 10.2%). CD 4 and 8 positive T cells make up a smaller contribution of endometrial lymphocytes compared to blood (28.3 vs 61.7%), and B lymphocytes constitute a much smaller proportion in the uterus (1.3% vs 12.9%).

Further analysis of the endometrial results revealed the data was not normally distributed for the majority of parameters (Fig. 3), so medians and centiles were selected as the most representative statistics, rather than means. The natural killer cells present in the endometrium could be differentiated into three distinct populations based on cluster differential marker testing: uterine/decidual natural killer cells (referred to as uNK, CD16−ve and $CD56^{bright}$), peripheral blood type (pNK, CD16+ve and $CD56^{dim}$), and natural killer T Cells (CD3+ve, CD16−ve, $CD56^{dim}$). The main lymphocyte in the endometrial environment was the uNK cell and comprised 46.9% of all lymphocytes on average (Table 3). Interestingly, the other types of natural killer cells (pNK and NK-T) were also present, but in much smaller numbers. Peripheral blood type natural killer cells were still detected in the prepared endometrium, but at a mean of only 1.6% of the total lymphocyte population, although perhaps significantly, they were present in much higher numbers in certain patients. B lymphocytes are also only present in small numbers in the endometrium (1.3%), but again, a small proportion of cases had levels well beyond this. T cells expressing CD4 and CD8 were seen to varying degrees in all cases, but interestingly, the ratio of CD4:CD8 was inverse to that seen in peripheral blood due to CD8 predominance.

Based on past medical history, the study group included 62 patients with a background of recurrent pregnancy loss (31%), and 64 with repeated implantation failure (32%). When the population was divided based on aetiology for subgroup

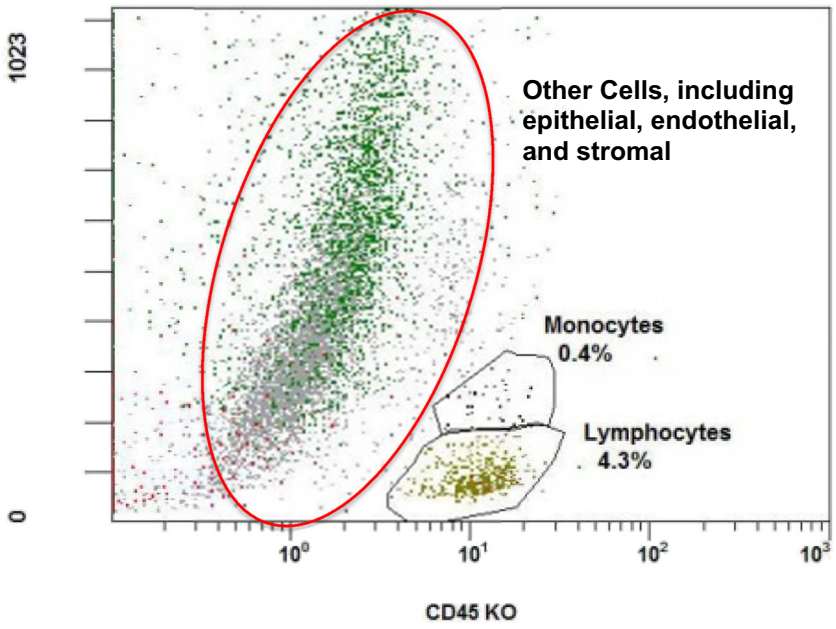
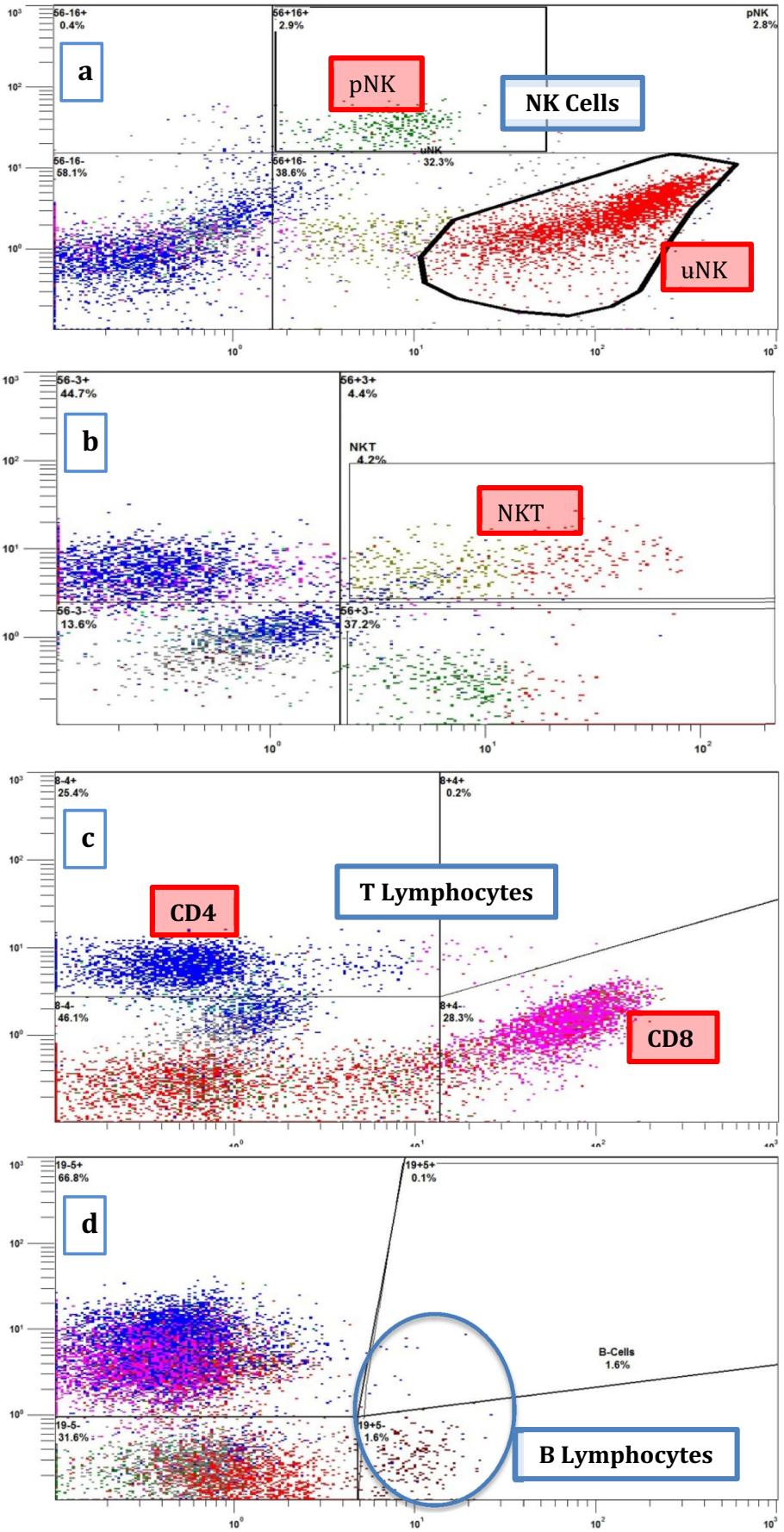


Fig. 1 Side scatter illustrating the distribution pattern of the CD45+ ve lymphocytes gate on FCM


a
56-16+
0.4%
56+16+
2.9%
pNK
2.8%
pNK
NK Cells
56-16-
58.1%
56+16-
38.6%
uNK
32.3%
uNK
b
56-3+
44.7%
56+3+
4.4%
NKT
4.2%
NKT
56-3-
13.6%
56+3-
37.2%
c
8-4+
25.4%
8+4+
0.2%
T Lymphocytes
CD4
8-4-
46.1%
8+4-
28.3%
CD8
d
19-5+
66.8%
19+5+
0.1%
B-Cells
1.6%
19-5-
31.6%
19+5-
1.6%
B Lymphocytes

◀ **Fig. 2** Further subdivisions of the designated lymphocyte gate using specific immunomarkers: **a** CD56, CD16; **b** CD56, CD3; **c** CD8, CD4; **d** CD19, CD5, clearly identifying target subsets. Backgating can be seen to colour cell populations in the neg-neg quadrants

analysis, several differences were apparent between the miscarriage and implantation failure groups when comparing medians using Mann-Whitney *U* test (Table 4). Patients with recurrent pregnancy loss had significantly higher levels of pNK (1.21 vs 0.80, $p = 0.041$), NK-T (2.68 vs 2.26, $p = 0.031$), and B lymphocytes (0.87 vs 0.72, $p = 0.032$). In the repeated implantation failure group, the uNK cellular proportion was significantly higher (52.4 vs 43.7, $p = 0.01$). No differences were observed in total levels of CD4 or CD8 T lymphocytes between these groups.

Using the 5th and 95th centiles to determine the population reference ranges, threshold values can be obtained for each parameter (Fig. 3 and Table 3). Using these levels, various trends can be identified. For example, when analysing uNK levels, 80% of those above the normal range (> 95th centile) had a background of RIF, while 60% of the low group (< 5th centile) presented with RPL (Fig. 4). Interestingly, recurrent miscarriage patients constituted 70% of those with elevated pNKs, and 90% of the patients with raised B cells. Continuing research and extension of sample collection to incorporate prospective analysis of endometrium from fertile controls, in addition to those with poor reproductive outcome, will improve the accuracy, reliability, and validation of the proposed normal ranges.

Discussion

The immune system is without doubt involved in the implantation process, and subsequent pregnancy loss. What is unclear is how pathophysiological changes in endometrial immune function correlate with clinical outcomes, if any diagnostic tests are sufficiently reliable/accurate to investigate this area, and whether any subgroup of patients exists that may benefit from adjunctive treatment.

Table 2 Mean percentage of immunological subtypes as a proportion of total CD45+ lymphocytes for endometrial ($n = 200$) and peripheral blood samples

Lymphocyte subtype	Endometrium Mean (%)	Peripheral blood Mean (%)
NK	48.5	10.2
NK-T	3.5	4.5
CD4+	11.4	40.7
CD8+	16.9	21.0
B cells	1.3	12.9

Attempts to study peripheral blood lymphocytes in the circulation as a marker of endometrial immune dysfunction have been highly controversial. Regarding natural killer cells for example, it has been suggested that there is little physiological reason to propose that a relationship between peripheral blood and endometrial levels should exist [14]. Interesting data has however been reported, and correlated with patient aetiology, when lymphocyte levels are studied locally in the endometrium. A 1996 study showed significantly decreased percentages of endometrial CD8+ T lymphocytes, and increased B lymphocytes in recurrent pregnancy loss patients [15]. Important UK data have demonstrated that women with recurrent miscarriage had significantly more uterine natural killer cells than controls, and that prednisolone treatment significantly reduced the number of endometrial CD56 cells [16]. Recent French research has showed that endometrial immune profiles were dysregulated in over 80% of implantation failure patients compared to controls, with the presence of both overactivation and also low activation [17]. The hypotheses of underactivation of the endometrial immune system could suggest a reason for the poor results from many studies using immunosuppressive treatments in unselected patients with recurrent miscarriage or implantation failure. There is certainly potential for endometrial assessment to have a role in the evaluation of carefully selected cases, but a greater understanding of the relevant constituent parameters, definition of normal ranges, and potential prognostic value is needed before this can be routinely offered to patients. This pilot study assesses if a locally developed, rapidly, qualitative flow cytometry technique can adequately detect the endometrial lymphocyte populations, determine putative normal ranges, and compare results between groups.

The role of the endometrial immune cells in cases of recurrent implantation failure, especially despite transfer of proven PGT euploid embryos, is particularly poorly understood, with proposed treatments for suspected abnormalities having a limited evidence base to justify their use. Recent meta-analyses have reported the lack of robust supporting evidence for pharmaceutical interventions such as intralipids, corticosteroids, or IVIG in the setting of implantation failure, adding to this highly controversial debate [18]. Conversely, some authors have reported that immunomodulation may actually enhance live birth rates, but only if used specifically in women displaying abnormal immunological risk factors [19]. A major issue with existing reproductive immunology studies is patient selection, particularly the lack of clear and consistent criteria for defining patient inclusion or exclusion. As the primary reason for ART failure is typically embryo aneuploidy, any potential endometrial intervention is likely futile unless a chromosomally normal blastocyst is transferred. If immunomodulatory treatments are to be properly assessed, then well-defined diagnostic entry criteria are needed to indicate women with a potential underlying alloimmune factor, and ideally incorporating PGT

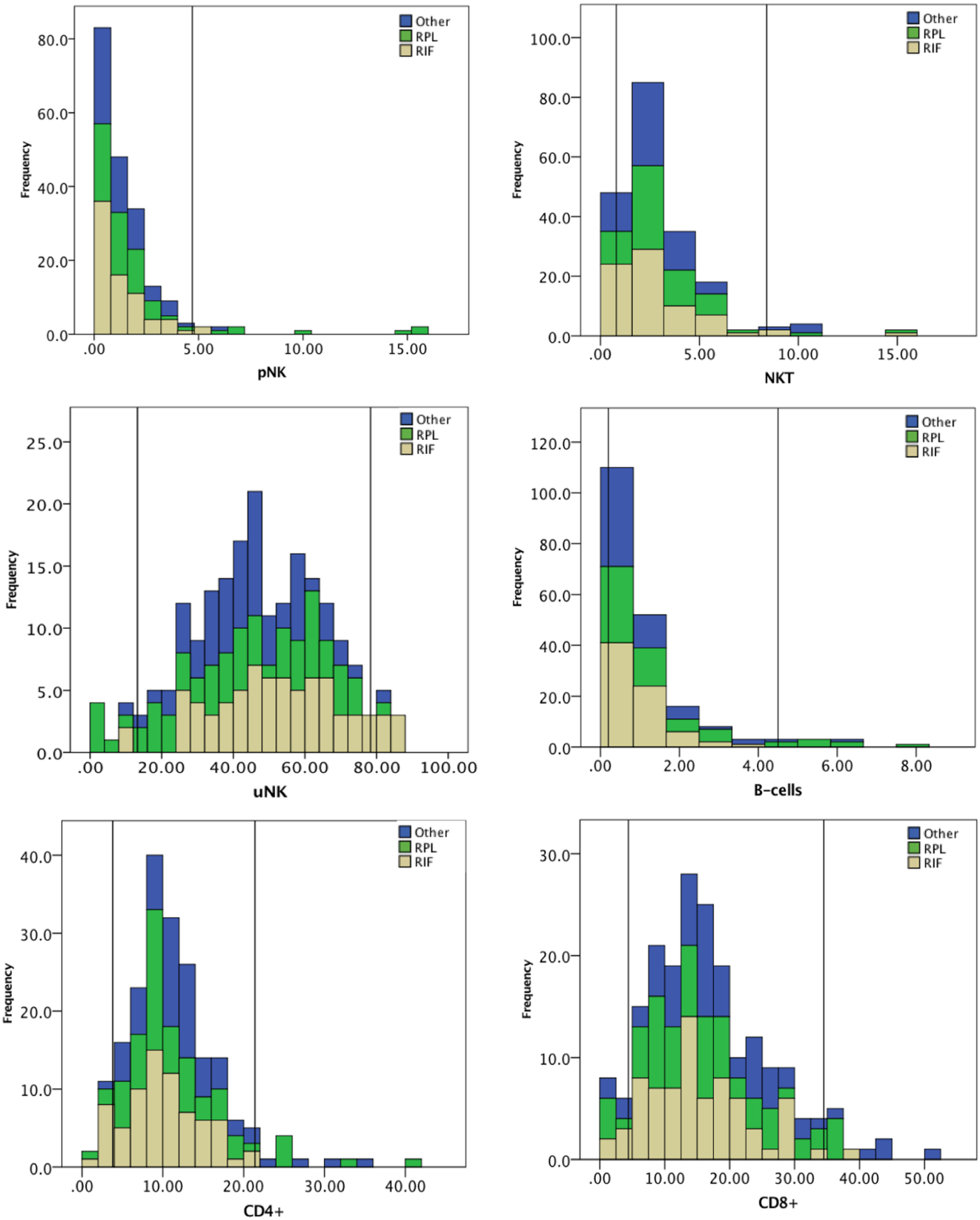


Fig. 3 Histograms demonstrating distribution of lymphocyte populations, with patient aetiology and 5th–95th centiles demonstrated

Table 3 Median percentage of immunological subtypes as a proportion of total CD45+ endometrial lymphocytes for all patients ($n = 200$) and proposed "normal" ranges

Lymphocyte subtype	Median	Range (5–95th centile)
uNK	46.0	13.2–78.3
pNK	1.0	0.0–4.7
NK-T	2.5	0.8–8.4
CD4+	10.4	3.8–21.4
CD8+	15.7	4.4–34.5
B cells	0.7	0.2–4.5

to confirm embryo normality and exclude aneuploidy as a confounding variable. In this situation, studies be designed with adequate statistical power to determine if immunotherapy treatments have a valid role for some cases, or are unnecessary and expensive adjunct.

Peripheral blood has been shown to be a relatively poor predictor of endometrial immune dysfunction, so localised uterine markers may be preferable to identify those patients at potential risk. Endometrial tissue assessment following an office biopsy is a moderately invasive technique, but benefits from direct evaluation of the compartment where implantation failure or miscarriage occurs, as opposed to the traditionally used blood sample, where the cellular populations are undoubtably different. Local endometrial levels of NK cells, B cells, and T lymphocytes could be better markers to determine who should be considered appropriate for interventional studies to assess the utility of personalised treatment. Improved interpretation of normal endometrial lymphocyte levels is therefore required in order to understand this further, and determine appropriate inclusion criteria. A targeted and detailed endometrial assessment could be used to identify patient subgroups with a poor reproductive who despite transfer of morphologically normal blastocysts also have a demonstrably abnormal uterine environment. This would allow development of well-designed prospective randomised trials to determine if specific and tailored immunotherapy treatments, personalised to the individual endometrial immunophenotype,

Table 4 Median percentage distribution of immunological subtypes as a proportion of total CD45+ endometrial lymphocytes between patient subgroups. *p* values determined using Mann-Whitney *U* test (SPSS v24)

Cell type	RPL (%)	RIF (%)	*p* value
pNK	1.21	0.80	*0.041**
NK-T	2.68	2.26	*0.031**
uNK	43.7	52.4	*0.010**
B cells	0.87	0.72	*0.032**
CD8+	15.1	14.1	*0.685*
CD4+	9.75	9.36	*0.168*

The italic entries with asterisk indicate statistical significance below $p = 0.05$

could actually have a beneficial role in these carefully selected populations, or if indeed there is truly no benefit whatsoever.

Altered uterine immune profiles have been both hypothesised and reported, but there is no clear and agreed definition regarding what is normal or abnormal. Variation in NK cell populations, as well as T and B lymphocytes, has been proposed as having an association with reproductive failure [14, 20–22]. uNK cells have previously been reported to constitute up to 70–90% of lymphocytes in the uterus [13]. Although they are often found in this range, this study data shows they constitute closer to 50% of lymphocytes on average when the endometrium is analysed shortly after collection by FCM, but with a wide variation in proportions between patients. While peripheral NK cells demonstrate cytotoxic activity and are actively involved in defence against infections and neoplasia, uterine NK cells have been shown to exhibit more limited cytotoxic activity [23]. There is no clearly defined mechanism for the appearance and differentiation of uterine natural killer cells during the mid-secretory phase of the menstrual cycle [13]. Theories have been proposed including recruitment of mature peripheral NK cells to the uterus, formation of uterine NK cells by differentiation of stem cells in the endometrium, and differentiation of peripheral NK cells into uNK cells [24–27]. The functions of uNK cells are regulated by both activating and inhibitory receptors binding to trophoblast HLA class I, and are involved in the regulation of trophoblast invasion, angiogenesis, and spiral artery remodeling [28]. More research will determine if a minimum amount of uNKs are required to support a pregnancy, or if there is an upper threshold, above which success rates will be affected. For further assessment of uNK function, additional biomarkers may need to be explored. For example, IL-15 and Fn-14 mRNA have been proposed as indicators of uNK activation and maturation [17].

This large pilot study demonstrates the feasibility and reliability of a rapid flow cytometric analysis for determination of a detailed endometrial lymphocyte immunophenotype. Population means, ranges, and centiles have been described, which provides useful information when analysing patient results, and determining inclusion levels for future interventional studies. Clear differences have also been shown between recurrent miscarriage and implantation failure patients, which may help investigate the underlying pathophysiology in these groups. The lack of a normal fertile control group is an unfortunate limiting factor. The cases all have a poor reproductive history, with repeated episodes of miscarriage or implantation failure, on a background of prolonged infertility. It is well known that the commonest reason for these outcomes is embryo aneuploidy, rather than localised uterine factors, so it is highly plausible that many of these "abnormal" cases should have a normal endometrium, and a proportion of the study population should therefore be representative of the normal uterine immunophenotype, with the extreme values likely

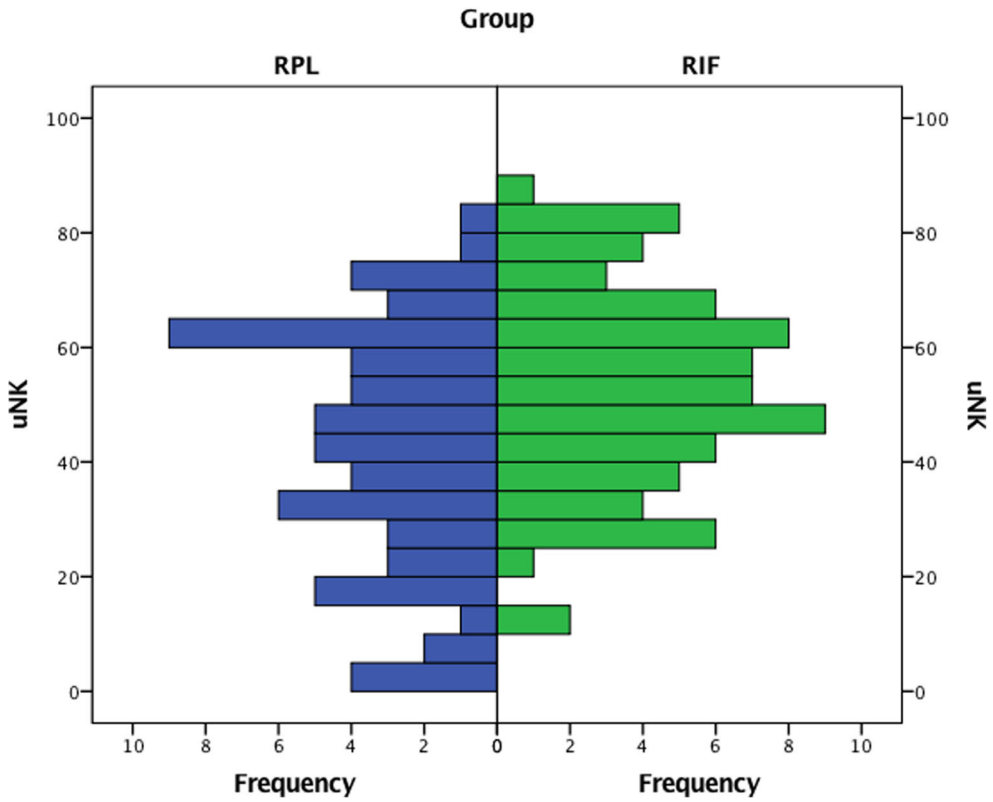


Fig. 4 Histogram demonstrating distribution uNK populations, in patients with RIF and RPL

indicating abnormalities. It is therefore anticipated that, even in the absence of specific fertile controls, the data allows determination of typical reference ranges of lymphocyte populations in the endometrium prior to implantation, and an assessment of what levels may be considered either elevated or reduced. Continuing recruitment to obtain greater patient numbers, and the important incorporation of a defined control group, will aim to corroborate these results. Another vital step is expansion of the lymphocyte subset analysis by inclusion of additional cellular markers to give a more in-depth assessment of extra cell types, providing more in-depth information and more answers going forwards.

As chromosomal screening of embryos prior to transfer becomes more accurate and utilised, there may be an increased demand for detailed endometrial assessment when proven euploid embryos do not give rise to an ongoing pregnancy. Molecular endometrial receptivity assessments are becoming more widely accepted, and can lead to specific personalisation of luteal support regimes in order to optimise identification of the peak window of implantation and timing of embryo transfer. Potentially, a more detailed immunological assessment may also have a prognostic role for these patients, tailoring therapeutic interventions to individual status, in order to optimise the environment for an embryo. A detailed assessment of the endometrial immunophenotype is an important starting point. Further study of this evolving area may help to further understand why chromosomally normal embryos may not implant or end with miscarriage, and identify therapeutic options to explore with a hope to improving success rates.

Compliance with ethical standards

Ethical approval All procedures performed were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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