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The distribution of immune cells and macrophages in the endometrium of women with recurrent reproductive failure I: Techniques

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ABSTRACT

Recurrent miscarriage affects approximately 1% of the population and in half of these cases no cause is found. Abnormally functioning immunocompetent cells, including natural killer (NK) cells, in the endometrium, are thought to be responsible for many such cases and treatment trials including oral prednisolone and intravenous immunoglobulins are now underway. Despite these encouraging developments, there is neither adequate standardization of counting uterine NK cells nor consensus as to what constitutes an abnormal level. To address this issue, immunohistochemistry was used to examine the presence and distribution of selected immune cells and macrophages in the endometrium from 222 women who had a routine endometrial biopsy for investigation of recurrent miscarriage or IVF failure, at various stages of the menstrual cycle, and accessioned prospectively over a 7-month period. Biopsies were examined by H+E and immunostained for $CD8^+$ T-cells, $CD163^+$ macrophages, $CD56^+$ NK cells, and $CD57^+$ cells. Cell numbers (expressed as immunopositive cells per mm^2) were determined in the stroma of the functional layer of endometrium and the relative concentrations of some cell types ($CD163^+$ macrophages, $CD56^+$ NK cells) were expressed as a percentage of all stromal cells. Routine H+E sections revealed 12 patients with focal "endometritis" without plasma cells. $CD8^+$ T-cells showed focal perivascular aggregates in most instances, and non-random but scattered cells in all cases, with a twofold increase in the luteal phase. $CD163^+$ cells were distributed evenly throughout the superficial endometrial stroma and also present as single or clustered macrophages within the lumens of superficial glands, mostly in the luteal phase. $CD56^+$ NK cells showed "diffuse" but variable distribution throughout the functional layer and perivascular aggregates of various sizes in two thirds of cases. Raw cell counts were low and relatively stable in the proliferative phase, but increased somewhat during the first half of the secretory phase, while in the second half of secretory phase they increased six to tenfold. Percentage counts rose from approximately 5% of stromal cells in the early part of the secretory phase of the cycle to over 35% in premenstrual endometrium. $CD57^+$ cells were present in very low numbers in most cases. The study illustrates the complexity and variability of immune

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cell infiltration of endometrium. We stress the need for strict counting protocols and attention to histological criteria if any immunological perturbations potentially responsible for recurrent reproductive failure are to be identified. Reference ranges for individual cell types are only valid for individual "days" of a normalized menstrual cycle.

1. Introduction

Repeated implantation failure is a distressing, traumatic, and expensive clinical phenomenon that encompasses both early pregnancy loss and failure of embryo attachment. The former is well recognized as the syndrome of recurrent miscarriage (RM), which affects up to 1% of the population and is conventionally defined as "three or more consecutive pregnancy losses prior to 20 weeks gestation" (Pandey et al., 2005). Women with RM may conceive naturally or by in vitro fertilization (IVF). As IVF has become a common and accessible treatment for a wide variety of indications, it has become apparent that some couples suffer repeated IVF failure (RIF). There is no consensus definition of RIF. Previous reports have suggested the failed transfer, for example, of at least ten cleavage-stage embryos (Tan et al., 2005) or five blastocysts (Rinehart, 2007). In those women, the repeated transfer of good quality embryos into the uterine cavity is an indicator of endometrial receptivity and a test for possible early implantation problems. Currently investigations of RM and RIF are similar, partly because they may have overlapping causes (Christiansen et al., 2006), and partly because there is no alternative.

The vast majority of miscarriages and failed IVF cycles are due to random genetic abnormalities in the embryo. Hence, few clinicians would investigate couples with one or two losses or failures. However, as such events occur more frequently, conventional investigations can identify possible underlying causes, such as uterine anatomical abnormalities, parental karyotype abnormalities, clotting disorders such as protein C deficiency (Dawood et al., 2003), and factor V Leiden mutation (Rey et al., 2003) and antiphospholipid syndrome (Tincani et al., 2003). In approximately 50% of couples with RM and RIF the problem remains "unexplained" after such investigation. While many of those will fall into the "unlucky" category of repeatedly abnormal embryo production and/or attachment, it is possible that some will have problems with the endometrium involving hormones, cytokines, growth factors, immunological factors, trophoblast features, and local leukocyte populations (Norwitz et al., 2001). A number of women experiencing recurrent fetal loss may have a specific defective immune response to the "semi-allogeneic fetal graft" (Dhont, 2003; Michimata et al., 2003).

Previous work suggests that maternal leukocyte populations in the endometrium of some women with recurrent miscarriages are significantly different from those carrying the fetus to term (Fukui et al., 1999; Lachapelle et al., 1996; Tuckerman et al., 2007) or associated with known fertility (Quenby et al., 1999). However, whether cell numbers are higher or lower in women with recurrent pregnancy failure depends on which of the endometrium, early decidua or peripheral blood is assessed (Wold and Arici, 2005; King et al., 2010).

The relationship between the resident populations of various immune cells and RM or RIF remains an experimental hypothesis. A conspicuous lack of basic data or even a standardized method of assessing the resident populations of these cells in the endometrium of women being investigated for this condition stands out as an important intermediate step in investigating the problem, at least by histological techniques. Routinely obtained histological material from a large number of women attending three Australian assisted reproductive technology clinical networks provided sufficient experience to enable development and illustration of a technique and to discuss observed pitfalls.

2. Materials and methods

2.1. Study sample

Two hundred and fifty-four women attending the clinics of Sydney IVF (Sydney), IVF Australia (Sydney) and Repromed (Adelaide and Darwin) for the investigation and management of RM or RIF, as defined above, underwent routine diagnostic endometrial biopsy (either formal dilatation and curettage or Pipelle endometrial sampling) to determine possible endometrial causes, and had formalin-fixed tissue referred to the GynaePath subspecialty unit of Douglass Hanly Moir Pathology in Sydney for histological assessment, over a 7-month period from August 1st 2008 to February 28th 2009. Of these, 222 samples were adjudged to represent histologically normal endometrium from various stages of a natural menstrual cycle with adequate material for assessment and accompanied by relevant clinical information confirming eligibility for the study. The other 32 cases were excluded, most usually because the patients were having programmed (artificial) cycles ($n = 10$). In other cases, the endometrium was regarded as abnormal (e.g., chronic plasma cell endometritis, polyps, hyperplasia, anovulatory, $n = 7$), the slides/paraffin blocks were not retrievable from external reference laboratories ($n = 7$), the specimen included decidua or products of conception ($n = 6$) or inadequate or poor quality material was submitted ($n = 2$). Histological focal "endometritis" (the presence of periglandular lymphohistiocytic infiltrates, but an absence of plasma cells on careful examination – see below) was not an exclusion criterion.

The majority of patients had been investigated for other maternal causes of recurrent pregnancy loss or IVF failure, such as parental karyotype abnormalities, maternal structural uterine abnormalities, antiphospholipid and familial thrombophilia syndromes, and genital tract infections. As

Table 1
Summary of primary antibodies and immunohistochemical technique.

Antibody	Source	Clone	Dilution	Antigen retrieval
CD8	Dako	C8/144B	1/100	CC1 (Ventana)
CD163	Novocastra	NCL-CD163	1/100	CC1 (Ventana)
CD56	Novocastra	NCL-CD564	1/50	CC1 (Ventana)
CD57	Novocastra	NK1	1/25	CC1 (Ventana)

patients were not subjected to any additional procedures to enable the study to be carried out, were not given interviews or questionnaires, and as therapeutic implications of results were neither sought nor offered, advice was received that there were no ethical issues relevant to this study.

2.2. Tissue processing

Biopsy material was fixed in 10% neutral buffered formalin and all was processed into paraffin within 48 h. Sections were cut at 4 μm and stained with hematoxylin and eosin (H + E) according to a standard protocol. Immunostains were prepared on serial sections from the same block, also cut at 4 μm and floated on SuperFrost Plus coated glass slides (Menzel-Glasner, Germany), and stained in an auto-immunostainer (Ventana Benchmark XT) using ultraView® DAB detection kit (Ventana Medical Systems). All staining batches included appropriate controls and employed commercially available monoclonal antibodies for $CD8^+$, $CD163^+$, $CD56^+$, and $CD57^+$ and immunohistochemical protocols as outlined in Table 1.

2.3. Histopathological assessment criteria for endometrial samples

All histological studies were performed by one specialist gynecological pathologist (PR). H + E stained sections were examined to determine the endometrial dating against a standardized 28-day cycle with ovulation, by convention, occurring at the end of the 14th day. The endometrium was dated on the most advanced changes present according to the protocol of Noyes et al. (1975), with variation from area to area of greater than 2 days being a potential cause for exclusion. Clinicians were generally encouraged to perform the biopsy in the late secretory phase with the intention of observing the adequacy of luteal phase changes. The presence and distribution of any obvious inflammatory cell infiltrates within the stroma or glandular lumens were noted.

Immunostains were perused, using the following study criteria:

1. Crude count of each cell type was undertaken in the stroma of the functional layer (defined for the purposes of the study as within approximately 1 mm of the identified endometrial surface in the curettage tissue fragments) and avoiding endometrial tissue from the lower uterine segment and from the basal layer. Fields for examination were not random, but were specifically directed to areas showing the most advanced endometrial dating and "average" (as opposed to highest or lowest) cell infiltrates.
2. Only immunostained cells in which whole nuclei could be identified ("circled" or obliterated by stained cytoplasm) were counted. Cytoplasmic fragments, extensions or dendritic processes were not counted. This was an important criterion since, when percentage figures were generated for some cell types, only whole nuclei (of stained or unstained cells) formed the denominator. Some cells characteristically showed cytoplasmic membrane staining, such as $CD56^+$ uNK cells, which frequently resembled "fried eggs," while others more typically exhibited dense uniform cytoplasmic immunoreactivity with obliteration of nuclei, such as $CD57^+$ cells and $CD163^+$ macrophages.
3. $CD8^+$ T-cells and $CD56^+$ uNK cells always showed both diffuse non-random stromal distribution as well as, quite frequently, perivascular or periglandular aggregates. Cell counts specifically excluded these aggregates (as they were impossible to count), but their presence and prominence were noted. This again meant that fields chosen for counting could not be random.
4. $CD57^+$ NK cells are common in the peripheral circulation and care is required to exclude $CD57^+$ cells that are within vessels in the endometrial stroma, as well as extravasated cells in areas of stromal hemorrhage (which were avoided). As double labeling was not available to us, it was not possible to discriminate between $CD57^+$ NK cells and $CD57^+$ T lymphocytes in the tissues.
5. $CD163^+$ macrophages showed both diffuse stromal infiltrates as well as, frequently, single or clustered cells with the lumens of superficial endometrial glands. These latter cells were not counted but their presence and prominence were recorded.
6. Stromal cell counts were expressed as cells per mm^2 by counting five high power fields (HPF) with 40× objective in stroma alone (approximately 1 mm^2) and rounding out to the nearest 10 cells per mm^2. When the count was obviously immeasurably large and more than 100 stained cells were counted in a single HPF, the total count was expressed as >500/mm^2.
7. Percentage cell counts were performed on a single oil immersion field (100×) of superficial stroma, adjudged at lower power to be representative (i.e., of "average" cell density) in each case. Immunostained cells, as defined above, were calculated as a percentage of all stromal cells (intact whole nuclei of stained and nonstained cells), counted in that single field. The total number of stromal cells per single oil immersion field varied from approximately 120 in the mid-secretory phase when maximal stromal edema was present, to about 160 in the early and late secretory phase, and a manageable number (e.g., a single HPF regularly contained >1000 cells).

Derivative data were tabulated according to the day of the endometrial cycle and mean values and 25th and 75th percentiles were calculated using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Pathological endometrial changes

The 222 endometrial biopsy samples were from women aged 24–45 years (mean 36 years). The endometria were dated to correspond to a standardized 28-day cycle, and their distribution according to this dating is given in Table 2. The vast majority of cases were either day 23 or 24 of the cycle, in accordance with the preferred timing, but inaccuracy in clinical dating and the exigencies of patient/clinician convenience gave a reasonable temporal spread of useful data regarding the whole cycle.

The routine H + E sections revealed little of note. To the extent that these patients had routinely been screened for genital tract pathogens and hyperplasias, plasma cell endometritis, luteal phase defects, and polyps were exclusion criteria, this finding was not unexpected. Nevertheless, in 12 patients, histological patterns of focal "endometritis" were observed, consisting of mixed lymphohistiocytic infiltrates around endometrial glands, frequently infiltrating the glandular epithelium and producing cellular aggregates within gland lumens, which sometimes included neutrophil polymorphs (Fig. 1a). These periglandular infiltrates proved to be $CD8^+$ T-cells ($n = 4$; Fig. 1b), $CD163^+$ macrophages ($n = 3$; Fig. 1c), or both cell types ($n = 5$). $CD56^+$ uNK cells also participated in these periglandular infiltrates to a variable extent (Fig. 1d). Plasma cells were not seen in the stroma of these cases (an exclusion criterion),

Table 2
Distribution of cases according to endometrial dating (against a standardized 28-day cycle with ovulation occurring at the end of the 14th day).

Dating	Number
Proliferative	11
Secretory	
Day 17	3
Day 18	10
Day 19	6
Day 20	8
Day 21	8
Day 22	9
Day 23	59
Day 24	61
Day 25	38
Day 26	6
Menstrual	3
Total	222

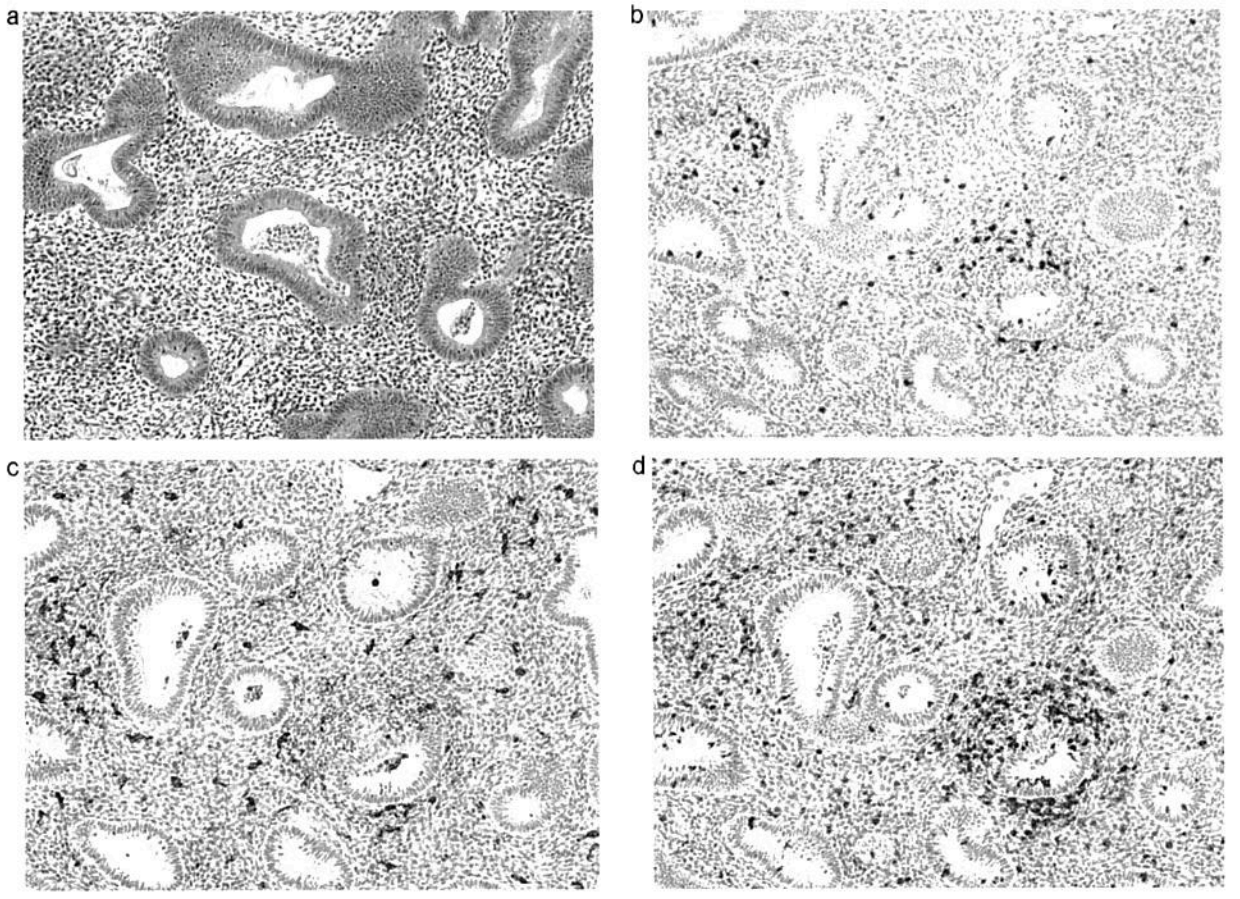

Fig. 1. Proliferative phase endometrium with a mild focal endometritis. A mixed mononuclear cell infiltrate cuffs the endometrial gland, with some cells infiltrating the glandular epithelium and residing within the gland lumen. (a) H + E 200×, (b) $CD8^+$ T-cells, (c) $CD163^+$ macrophages, and (d) $CD56^+$ uNK cells.

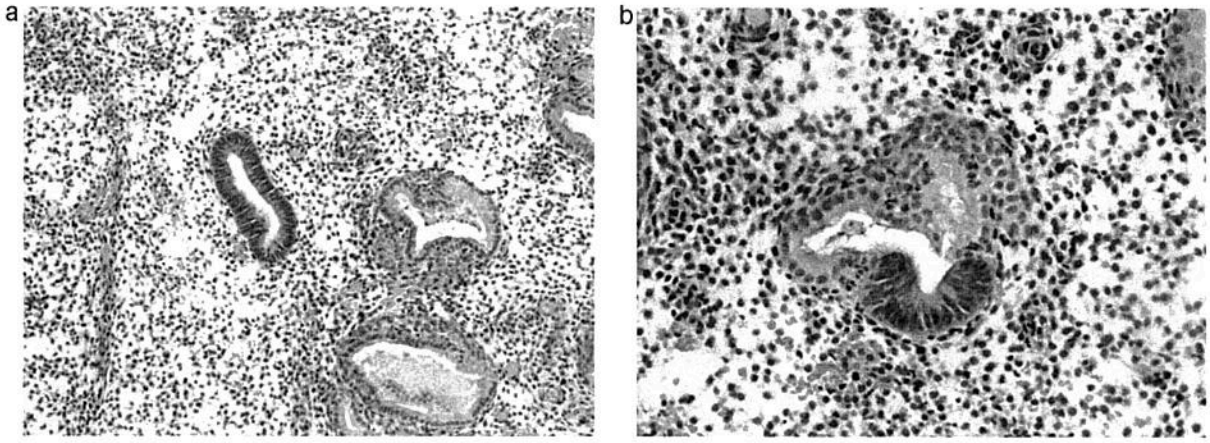

Fig. 2. Conspicuously nonsecretory glandular epithelium in otherwise normal late secretory endometrium. This change was seen in either entire glands (a) or, in some instances, in segments of glands (b). H + E 200×.

confirmed by CD138 immunostains. Yet, we have anecdotal evidence of a few such patients given broad spectrum antibiotics (Augmentin), with resolution of the process in follow-up curettage specimens.

Another observation, not exclusive to this group of patients, but seen with some regularity, was the presence of solitary or clustered endometrial glands which, despite advanced (progesterone-induced) secretory phase changes in adjacent glands and stroma, exhibited a conspicuous lack of such cellular alterations. Usually, entire glands were affected (Fig. 2a), but sometimes only a segment of a gland showed this change (Fig. 2b), and presumably on the basis of focal aberrations in the receptor status of the glands.

3.2. CD8 cell counts

Immunohistochemically, $CD8^+$ T-cells were readily assessable. The immunostaining was robust, specific, and varied little in its intensity between cases or at different times of the cycle. Two patterns of cell distribution were apparent – focal and "diffuse." As noted above, focal perivascular aggregates were present in most instances, frequently only small, but sometimes large and dense, the latter completely obscuring the constituent cells (Fig. 3). Focal periglandular concentrations were also present in a small number of cases (Fig. 1b). With participation of other cell types, infiltration of the glandular epithelium and the presence of cells or debris in the gland lumens, this was interpreted as a focal "endometritis" (i.e., inflammation in the endometrium – see above). We have observed three such patients in this series in whom the change was not present in follow-up curettage specimens after broad-spectrum antibiotic therapy. Perivascular aggregates were more common in patients in whom the diffuse stromal population was higher, but this was not always the case. Thus, the routine in this study was to count the scattered $CD8^+$ cells in stroma away from perivascular collections, but to note their presence and prominence. Care was taken to ensure that variation from area to area was taken into account (Fig. 4a) and, to this extent, chosen HPFs were not strictly "random." The count also did not include the frequently observed, single intraepithelial cells, which were unrelated to the periglandular aggregates described above. With this protocol, a twofold increase in cell counts was observed from about 70 cells per mm^2 at ovulation to 140 cells per mm^2 prior to menstruation (Fig. 4b), although significant variation was observed between cases (Table 3). The "dip" around days 21–22 was understandably a function of stromal edema. There was no further increase with the onset of menstruation.

3.3. CD163 cell counts

$CD163^+$ tissue macrophages also manifested two separate and distinct dominant patterns of distribution in addition to a third minor pattern. $CD163^+$ cells, in the stroma, were distributed fairly evenly throughout the superficial layer of the endometrium (Fig. 5a) with no tendency toward perivascular aggregation. Cell counts, at about 160 cells per mm^2 (Table 3) and percentage values at about 4% of endometrial stromal cells were stable

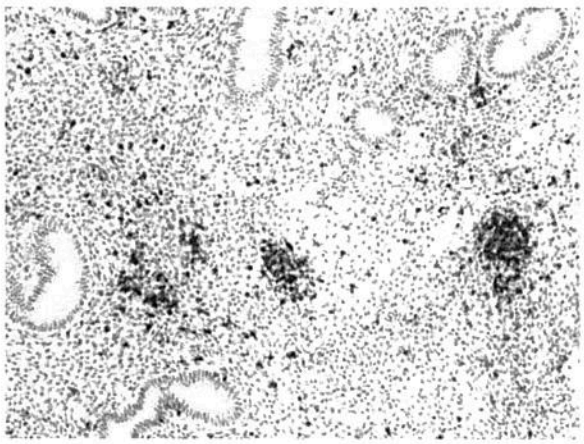

Fig. 3. Perivascular aggregates of $CD8^+$ T-cells in the late secretory phase. The size and density of these cell collections precluded any sensible attempt at counting the constituent cells present.

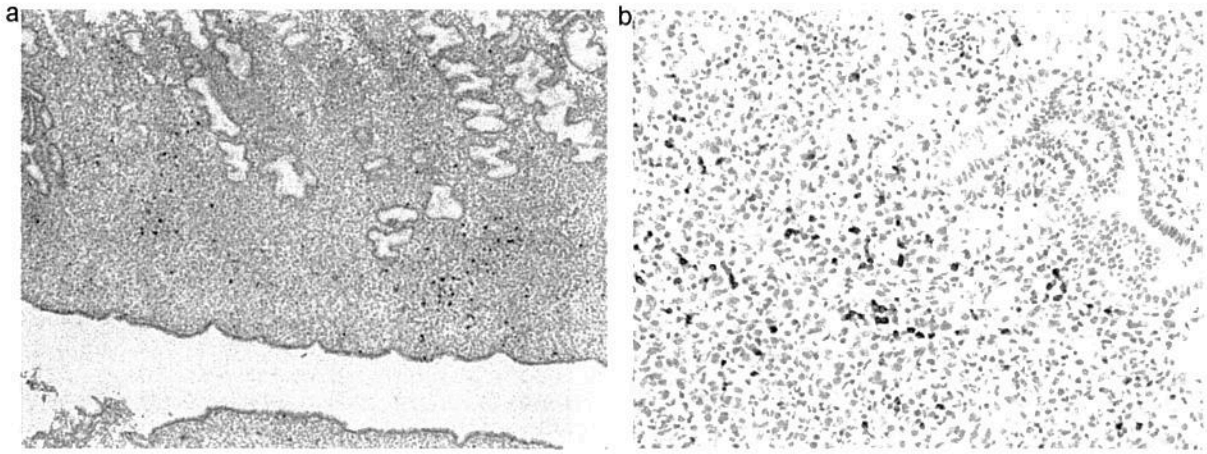

Fig. 4. (a) CD8⁺ T-cells in day-24 endometrium with obviously increased numbers in the vicinity of the spiral arterioles. (b) Scattered distribution of CD8⁺ T-cells in the stroma of early menstrual endometrium from a patient with recurrent miscarriage.

throughout the proliferative and early-to-mid secretory phase, while both increased only in the late secretory phase to about 320 cells per mm^2 (Fig. 5b) and 7% of stromal cells (Fig. 5c), immediately prior to menstruation.

The second common pattern was the presence of single or clustered CD163⁺ macrophages within the lumens of superficial endometrial glands – a pattern identified in 175 of the 222 cases overall (79%). This process was seen

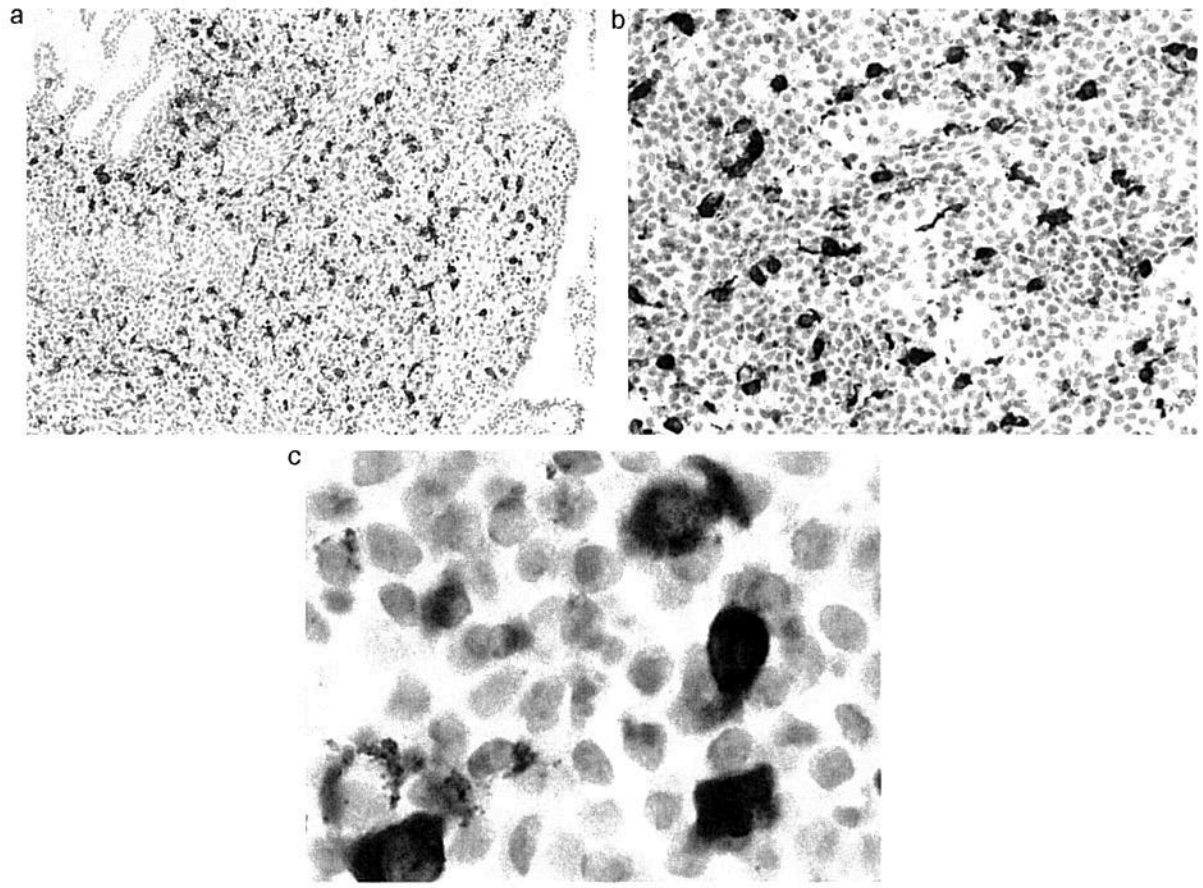

Fig. 5. CD163⁺ tissue macrophages within the superficial endometrial stroma of women with recurrent miscarriage. (a) Low power to show relatively even distribution (10×). (b) High power (40×), at which raw cell counts per mm^2 were made, highlighting the importance of counting immunostained cells in which a whole nucleus can be seen and (c) oil immersion (100×) at which percentage counts were assessed.

Table 3
Mean CD8, CD163, CD56, and CD57 cell counts per mm² in the superficial endometrial stroma, according to endometrial dating.

	PP	17	18	19	20	21	22	23	24	26	26	MP
CD8	100	80	100	110	105	65	120	110	120	110	150	–
CD163	160	250	130	195	205	160	160	180	250	305	270	–
CD56	120	40	90	245	210	170	290	440	1075	1190	1655	–
CD57	20	20	20	0	0	15	10	0	0	5	20	–

PP = proliferative phase, 17–26 = secretory phase days, MP = menstrual phase.

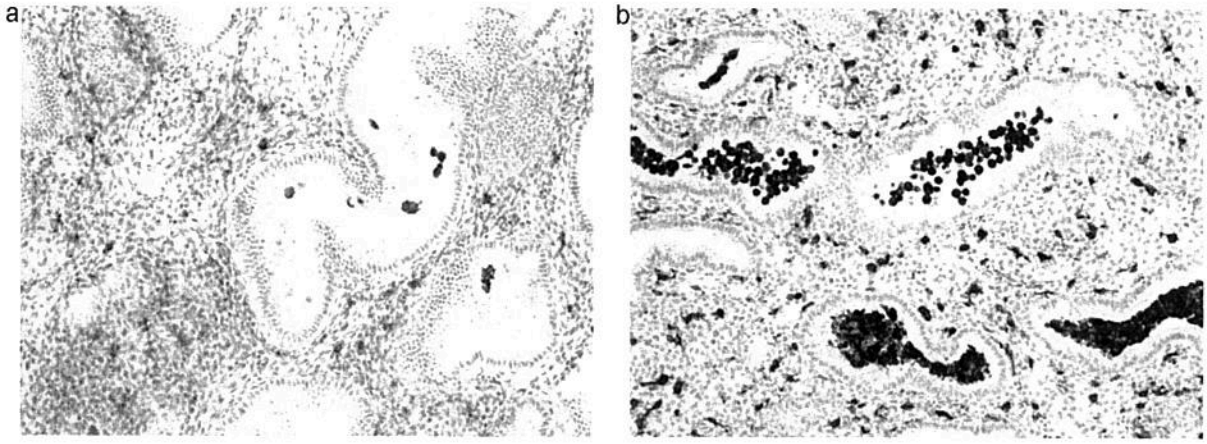

Fig. 6. CD163⁺ macrophages within the lumens of superficial endometrial glands. This was a very variable but commonly encountered process, the examples illustrated here being amongst the most mild (a) and most intense (b). This process was separate and quite distinct from the occasional case in which CD163⁺ macrophages cuffed endometrial glands as part of a focal endometritis.

in only 4 of the 11 proliferative phase cases (and was very mild in all such cases), but varied in intensity, in the secretory phase, from occasional glands containing solitary macrophages (Fig. 6a) up to many glands containing large numbers of macrophages or even confluent masses (Fig. 6b). The glands containing intraluminal macrophages often appeared, in the immunostains, to have inspissated mucus but were otherwise unremarkable. The process did not have any obvious relationship to the presence of red blood cells in gland lumens, another commonly encountered phenomenon of unknown etiology (Fig. 7). It bore no relationship to the focal "endometritis" already described, and neither was it associated with any alteration in the even distribution pattern or percentage concentration of macrophages in the stroma. The third and minor pattern, identified in only eight cases, was the participation of CD163⁺ macrophages in the periglandular cuffing of focal endometritis (Fig. 1c).

3.4. CD56 cell counts

CD56⁺ uNK cells were a major focus of this study. The monoclonal antibody employed gave reliable and

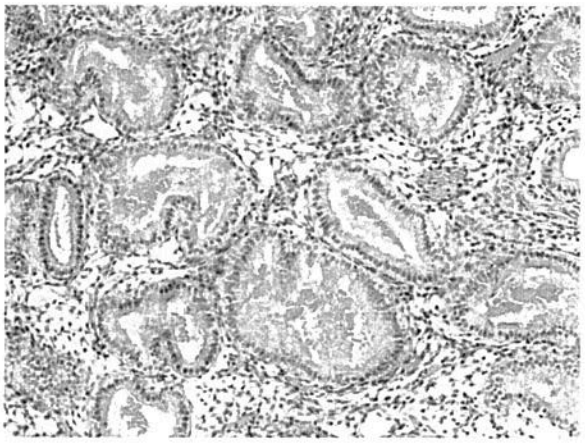

Fig. 7. Red blood cells within gland lumens in mid secretory phase endometrium. This was not infrequently encountered and was considered a possible but unlikely explanation for the aggregation of CD163⁺ macrophages within endometrial gland lumens (Fig. 6). H + E 200×.

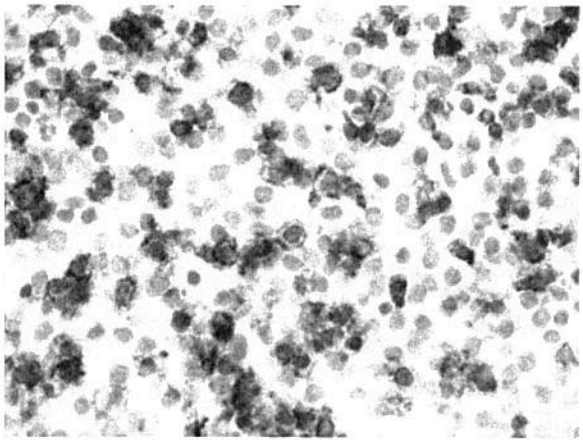

Fig. 8. CD56⁺ uNK cells, distributed evenly and in large numbers in the late secretory phase endometrium of a woman with recurrent miscarriage. This image is taken using 40× objective and would approximate to about 1500–1600 cells per mm².

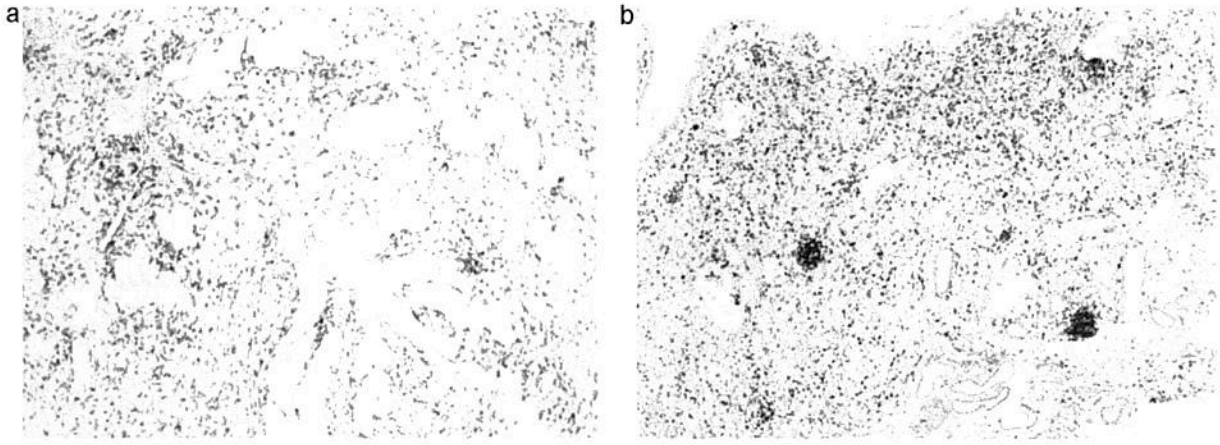

Fig. 9. Commonly encountered patterns of CD56⁺ uNK cells in recurrent reproductive failure. (a) Outlining the endometrial glands but not infiltrating the epithelium, (b) in about two thirds of cases, there was a tendency for the cells to aggregate around small vessels.

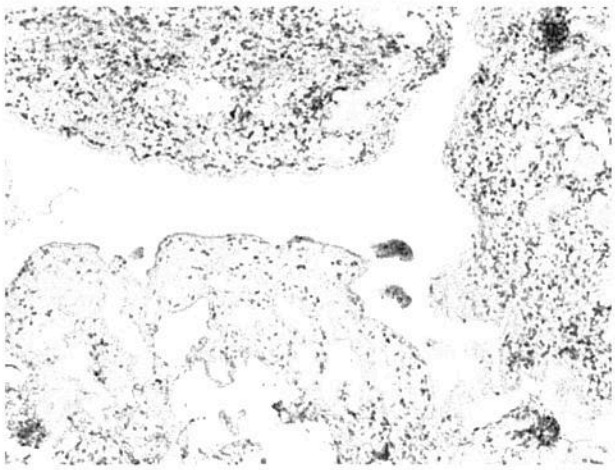

Fig. 10. CD56⁺ uNK cells in recurrent reproductive failure. Variation from area to area within the superficial or functional layer of the endometrium requires some care to choose a representative or "average" field in which to count.

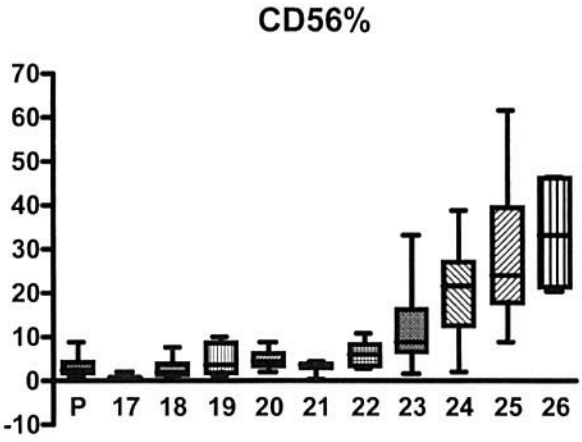

Fig. 12. Counts of CD56⁺ uNK cells as a percentage of endometrial stromal cells, showing mean counts and ranges. Hatched areas for each are between the 25th and 75th percentiles.

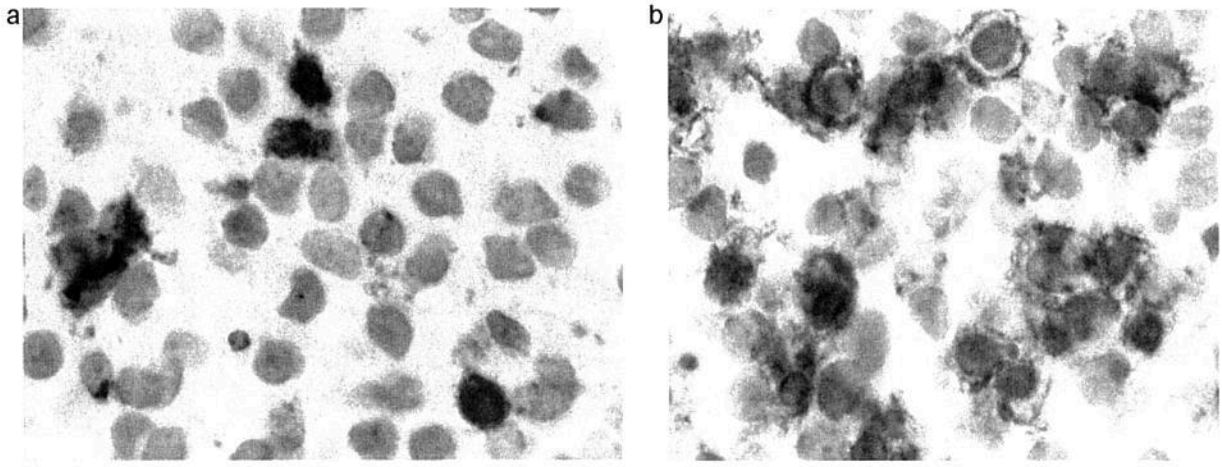

Fig. 11. CD56⁺ uNK cells in recurrent reproductive failure. (a) At day 19 of the cycle, the mean percentage of reactive cells was about 5% of all stromal cells in the superficial endometrium while (b) at day 26 of the cycle, the equivalent percentage was 33%. Note, in (b), the tendency of these cells to form small clusters of 2–4 cells.

robust immunostaining that did not vary during the cycle. Immunostained cells were generally distinguished by peripheral membrane staining to give a "fried egg" appearance (Fig. 8). CD56$^+$ cells had a similar distribution pattern to that of CD8$^+$ T-cells, namely "diffuse" or scattered distribution throughout the functional layer of the endometrium in all cases, "lining up" alongside the glandular epithelium in several cases (Fig. 9a) and producing perivascular aggregates of various sizes in approximately two thirds of cases (Fig. 9b). This latter feature was not more obvious as the density of CD56$^+$ cells increased during the late luteal phase. Although "mapping" was not generally undertaken in serial sections, the impression, in a few cases in which this was specifically sought, was gained that CD56$^+$ cells and CD8$^+$ cells were co-located around some vessels only. As with CD8$^+$ T-cells, the raw cell count was performed away from such aggregates and, again, care was taken to ensure that fields with "average" cell numbers were counted, particularly when numbers were low (Fig. 10). These numbers were, indeed, relatively low and relatively stable in the proliferative phase and the first half of the secretory phase, averaging approximately 100–250 cells per mm^2. In the second half of the secretory phase, however, they increased in number dramatically, to a mean count estimated to be over 1600 cells per mm^2 (Fig. 8) in premenstrual endometrium (Table 3) – a spectacular six- to tenfold increment over a mere week. The percentage count of these cells rose correspondingly, over the same period, from a mean of about 5% on days 19–22 of the cycle (Fig. 11a), to over 35% in premenstrual endometrium. Fig. 12 shows the mean percentage as well as 25th percentile and 75th percentile values for these cells. It is emphasized that the oil immersion field chosen at low power, for the purpose of performing percentage counts, should be "average" or representative, rather than random, as quite considerable variation was apparent from area to area in individual samples. This increase was accompanied by a tendency for these CD56$^+$ cells to form small groups (Fig. 11b) in contrast to distributed single cells earlier in the cycle. This complicated counts later in the cycle, as did the tendency for CD56$^+$ uNK cells to be bilobed, necessitating percentage counts under oil immersion. Apoptosis, common in CD56$^+$ uNK cells in premenstrual and menstrual endometrium, was manifest more by cytological dissolution (also making counting difficult) than by either nuclear pyknosis (present, if specifically sought, but usually inconspicuous) or karyorrhexis (rarely noted).

3.5. CD57 cell counts

CD57$^+$ cells presented a particular difficulty in counting, in that few cells were present in most cases and some which appeared at low power to be in the stroma, on closer examination proved to be within blood vessels (i.e., the peripheral circulation). Sometimes this was perfectly obvious (Fig. 13a), while in other instances more care was needed (Fig. 13b) not to inflate the count by including such cells. Care was also required not to count in areas of obvious stromal hemorrhage (lest the numbers also be inflated by CD57$^+$ NK cells derived from the blood) or tissue from the basal endometrium where CD57$^+$ cells were also regularly present in small perivascular clusters. The pattern of distribution in the functionalis was random and sparse. The cells were small and moderately to densely immunostained. No trend was observed in either absolute numbers (Table 3) or percentage counts throughout the cycle. Because numbers were generally very small, they were inordinately influenced by occasional cases with quite high CD57$^+$ cell counts (Fig. 13c). The mean count throughout the cycle was about 10 cells per mm^2.

4. Discussion

Women with recurrent reproductive failure deserve investigation and often need (and sometimes demand) an innovative approach. It is not unreasonable to consider the immunological basis of implantation as a possible cause of their repeated failures. Previous studies have caused considerable debate and controversy (Moffett et al., 2004; Rai et al., 2005), although there certainly do appear to be changes in CD56$^+$ uNK cell numbers in women with recurrent reproductive failure. It is unclear whether the changes reported so far (Quenby et al., 1999; Tuckerman et al., 2007; Yamada et al., 2003) are reproducible and of any clinical significance. We set out to assess whether the immunohistochemical analysis of endometrial biopsy specimens could be done reliably and consistently, and whether a manageable protocol could be established. Counting in specific tissue regions and establishing precise counting protocols manually are essential prerequisites for using automated image analysis to its full benefit. Similarly, setting the appropriate color thresholds and selection of the areas of interest still have to be done manually. Future studies with automated image analysis are being developed.

The biopsy specimens were obtained by either curettage under general anesthetic, or as an outpatient procedure using a Pipelle sampling device, as part of their investigation of recurrent miscarriage or IVF failure. Although it is a discomforting and invasive procedure, there is provocative evidence that such a procedure in itself can be therapeutic. Biopsy in the month before an IVF cycle increases cycle success rates (Barash et al., 2003). So, whether additional information on immune cell subsets is used clinically or not, the woman should still benefit from having the biopsy done.

Few immune cells, overall, are present in the proliferative phase of normal endometrium (King et al., 1998). Almost none are B lymphocytes, and the majority are T-cells, macrophages, and uNK cells (Laird et al., 2003). The number of lymphocytes steadily increases until the late secretory phase at which time there is a spectacular increase in CD56$^+$ CD16$^-$ CD57$^-$ uNK cells, which constitute about 75% of all stromal lymphocytes (Hill and Choi, 2000; Jones et al., 1998). The broad cell types discussed in the following paragraphs were, thus, the subject of a detailed examination of their presence, numbers, and patterns of distribution in the endometrium throughout the menstrual cycle.

CD8$^+$ cells are subdivided into Tc1 and Tc2, each producing a similar profile of cytokines as Th1 and Th2 respectively (Akdis et al., 1999; Michimata et al., 2002), and are involved with cytotoxic immune reactions. In the endometrium

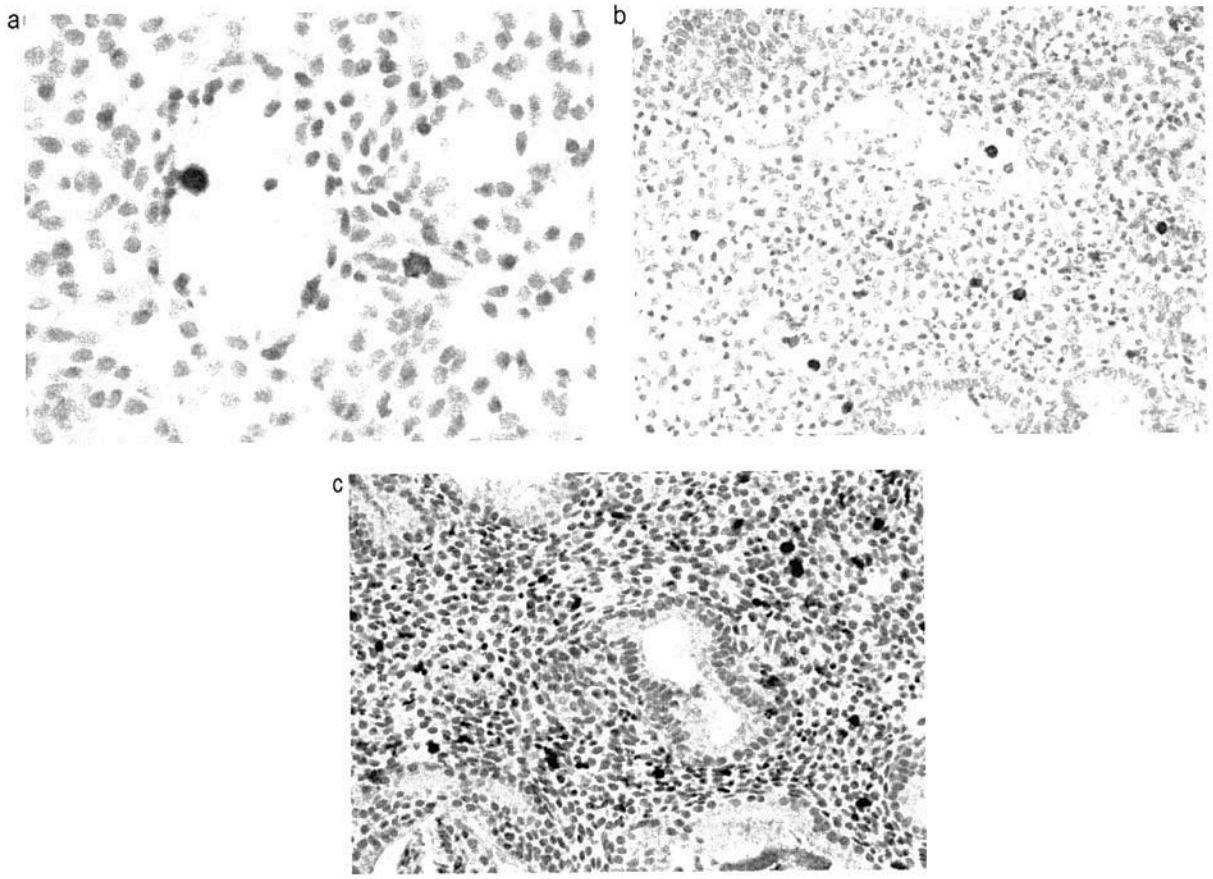

Fig. 13. CD57⁺ cells in recurrent reproductive failure. (a) Late secretory phase endometrium exhibiting two immunostained cells, the one on the left clearly in a dilated blood vessel and the one on the right in the stroma. (b) Menstrual phase endometrium with several CD57⁺ cells, some of which are in vessels (centre and lower left) and should not be counted. (c) High counts were occasionally encountered, as here, throughout the menstrual cycle.

they were regularly seen as perivascular lymphoid aggregates, as single cells within the stroma, or as scattered solitary intraepithelial lymphocytes, precisely mirroring earlier observations (Yeaman et al., 2001). While the precise function of the CD8⁺ cells is largely unknown in the endometrium, their function is reduced in the presence of IL-2 from Th1 cells (Yeaman et al., 2001). They are thought to play little if any role in the process of classical chronic endometritis, compared with the role of B lymphocytes (Disep et al., 2004; Tawfik et al., 1996), yet it is difficult to interpret quite intense focal periglandular aggregation of these cells, especially when accompanied by other inflammatory cell types such as macrophages and CD56⁺ uNK cells (see below), in any other way than as an occult focal "endometritis" or inflammation of the endometrium, despite the absence of plasma cells. Whether this is infective or non-infective is unclear. Examples of this process demonstrate aggregation around and into the glandular epithelium and leading to luminal accumulation (Fig. 1). While the late luteal acceleration in CD8⁺ numbers was synchronous with that of CD56⁺ uNK cells, it was nowhere nearly as pronounced. Yet perivascular aggregation did seem to co-locate these two immunocompetent cells in some but not most cases, and this impression is to be investigated further, perhaps using a double-immunostaining technique.

Tissue macrophages are reactive to a number of immunomarkers, but those most commonly used are CD68⁺ and CD163⁺ (the latter also known as hemoglobin scavenger receptor) (Davey et al., 1990; Madsen et al., 2004; Nguyen et al., 2005). Of these two markers, CD163⁺ is more readily interpretable in our hands and is more specific for macrophages than CD68⁺, which also stains myeloid cells, mast cells, and some B- and T-cells (Nguyen et al., 2005; Vakkila et al., 2005). CD163⁺ is a transmembrane glycoprotein responsible for the clearance of hemoglobin and as an anti-inflammatory associated with cytokine production (Aoki et al., 1995; Paidas et al., 2004). The number of CD163⁺ positive cells in plasma is known to increase in early normal pregnancy (Paidas et al., 2004), and a subpopulation of B7-H4⁺ immunosuppressive macrophages in the endometrium is thought to assist in establishing maternal immune tolerance against fetal antigens at the outset of implantation (Wicherek et al., 2009). The twofold increase in numbers of CD163⁺ macrophages late in the luteal phase in this study is consistent with both of these reports.

The observation, in the majority of biopsies carried out in the secretory phase, of CD163+ macrophages within

the lumens of superficial endometrial glands is puzzling. This has previously been noted briefly (Bulmer et al., 1988; Disep et al., 2004) in the context of both normal endometrium and of endometritis, but not further explored. In the present study it was common but variable, essentially confined to the luteal phase (occurring in almost 80% of such cases), often quite focal, and unassociated with any obvious pathogenetic features. There was some suggestion that the luminal mucin was different in the affected glands (more densely eosinophilic) than nearby unaffected glands and a plausible explanation is that of a macrophage response to modified gland secretions or contents (e.g., extravasated red blood cells, which are sometimes present in the glands of the functionalis in the luteal phase). Unlike the $CD8^+$ infiltrates, which seemed to respond to antibiotics (see Results), we have observed several cases in which the intraglandular accumulations of $CD163^+$ macrophages were equally severe in follow-up curettage specimens after broad-spectrum antibiotic therapy. This is also to be subject to further investigation.

Uterine NK cells have distinctive expression of monoclonal antibodies (Stockinger et al., 1996). The so-called $CD56^{bright}$ cells (on flow cytometry) represent the major population (Perez et al., 2003; Warren, 2000a). They can be detected by immunohistochemistry (as in this study), flow cytometry (Lachapelle et al., 1996; Maruyama et al., 1992) or electron microscopy (where they show increased cytoplasmic granules containing perforin and granzyme B) (Choudhury and Knapp, 2001; Konno et al., 1999).

Immunohistochemical assessment of tissues lacks the sophistication of flow cytometric analysis in which $CD56^{DIM}$, $CD56^{BRIGHT}$, and $CD56^{SUPERBRIGHT}$ subsets can be distinguished (Lukassen et al., 2004) and multiple antibodies can be used simultaneously with different fluorochromes to give double (or triple) labeling. The accentuated perivascular location of $CD56^+$ uNK cells described in this study has been reported previously, and it has been proposed by some groups that they might play a role in angiogenesis (Hazan et al., 2010) via remodeling of spiral arterioles (Croy et al., 2003). Indeed, these cells have been demonstrated to elaborate various growth factors necessary for angiogenesis, such as vascular endothelial growth factor (Lobo et al., 2004), but, for the purposes of this study, perivascular aggregation merely complicates the process of counting. Immunohistochemical studies of the $CD56^+$ uNK cells in the endometrium of women with recurrent reproductive failure are small and few, and contain even smaller control groups (Clifford et al., 1999; Quenby et al., 1999; Tuckerman et al., 2007). The changes that occur in $CD56^+$ uNK cell numbers in the second half of the luteal phase are so profound that, unless study and control groups are very carefully matched for stage of cycle (which can be problematic in some cases (Myers et al., 2004)), the statistical analysis is rendered meaningless. Similarly, if therapeutic decisions are ever to be based on derived cell counts, counting protocols need to sufficiently robust and explicit to be usable in different units and these are absent in the literature from even the most prestigious units (Quenby et al., 1999). This unit has suggested a cut-off of as little as >5% NK cells in mid-secretory endometrium as abnormal, warranting a trial of immunotherapy, and based on an interquartile range of 18 control patients (Quenby et al., 2005). Our study demonstrates 75th percentiles of uNK cell counts of 16%, 27%, and 39% respectively for days 23, 24, and 25 of the menstrual cycle in over 200 patients specifically identified as having recurrent implantation failure. Such massive changes in $CD56^+$ uNK cell density over the course of these days were not unexpected, but highlight the absolute necessity of interpreting results in the context of the particular histological date of the endometrium. Earlier in the cycle, when $CD56^+$ uNK counts are relatively stable, more comfortable comparison between published series is possible and the percentage counts we obtained at day 21, for example, are similar to those reported by Quenby et al. (1999), but significantly greater than those obtained by Clifford et al. (1999). It is crucial, therefore, to date the endometrium carefully (as noted above) and to standardize the selection of tissue fields for counting, as variation from area to area invalidates "random" selection for fields (lest a field contains a perivascular aggregate, or is from lower uterine segment tissue or basalis). Percentage counts on anything other than oil immersion fields are totally impractical, with care especially required in the late secretory phase when $CD56^+$ uNK cells tend to aggregate, tend to have bilobed nuclei, and are prone to apoptosis.

Finally, $CD57^+$ (HNK1) is also expressed on NK cells, an expression that is partially shared with some T-cells (Brinkmann and Kristofic, 1997; Stockinger et al., 1996). It is important in the development of the nervous system; however, its functional significance when expressed on NK cells is currently unknown (Warren, 2000b). The majority of cases (159 out of 222 – 71.6%) in the present study exhibited no or very few $CD57^+$ cells in the superficial endometrial stroma, an observation dependent upon excluding those cells in the peripheral circulation or in areas of stromal hemorrhage. Furthermore, as double labeling was not available to us, we could not discriminate between $CD57^+$ NK cells and $CD57^+$ T helper cells (the latter known to produce Th1-type cytokines and potentially mediating a transition from a pregnancy-supportive Th2 environment to a pregnancy-hostile Th1 environment). These observations are consistent with the literature, which further proposes that their presence is nevertheless inimical to implantation (Vassiliadou and Bulmer, 1996; Winger, 2007).

The genesis of this project commenced 7 years ago, by one pathologist (PR), firstly using manual immunostaining methods and with no detailed protocol in the published literature to follow. As noted above, we believe the latter contingency still obtains. In two subsequent iterations, different automated immunostaining platforms were used (presently the Ventana Benchmark XT system) for improved accuracy and standardization of immunohistochemical results (Bankfalvi et al., 2004). Over time, counting parameters were refined to those outlined in Section 2. It is a time-consuming exercise that requires firm adherence to the protocol to ensure consistency within and between cases in this series, and it is fatuous to assume that the data compiled can be directly compared with those of other published studies. Indeed, we believe that the absence of detail in the method of counting cells is the main

cause for variation in the reported results thus far, the other being the demonstrable variation with the stage of the cycle examined. "Apples with apples" comparison between control and study groups must, of necessity, include individual days of the cycle. We stress that the proliferation of uNK cells in the endometrium is highly dependent on the day of the cycle and must be interpreted accordingly. Based on the findings of our study, we find that giving a single figure of uNK cells per mm^2 or percentage of uNK cells, without the corresponding cycle date, is no longer an acceptable method of reporting these cases. Laboratories that intend to offer this test should develop their own in-house database.

The present study was confined to interval endometrium rather than miscarriage material. First, this is because it is manifestly unclear to us whether to measure cell numbers at or near the placental implantation site (not always present in the biopsy sample), or in the background decidua away from the placental implantation site. Second, the changes in such interval endometria might be of value in predicting future pregnancy prospects rather than identifying causes of previous pregnancy losses. The later date in the cycle to aim for with the biopsies (day 24 or LH + 10 rather than day 21), and where most of our data lie, is currently standard in order to include endometrial responsiveness to progesterone as a histological feature for assessment in this group of women. We have also set to one side the accumulated data on a number of patients having "programmed" cycles and are yet to analyze these to establish similarities or differences from those women with natural or spontaneous cycles.

The current study started out as a "proof-of-principle" exercise to investigate the feasibility of assessing various immunologically competent cells in endometrium, immunohistochemically. It sought specifically to address the numbers of $CD56^+$ uNK cells in interval endometrium obtained by formal dilatation and curettage or office-based endometrial sampling, in a large population of women otherwise being investigated in our laboratory for recurrent miscarriage or IVF failure. $CD8^+$, $CD\ 57^+$, and $CD163^+$ expressivity of endometrial immune cells were also examined. A method has been outlined that can be used as a basis for further study, such as the examination of regulatory T-cells or the presence of various cytokines such as TWEAK and the predominantly cytotoxic KIR receptor (Petitbarat et al., 2010; Wang et al., 2010).

There is some evidence that some women with repeated reproductive failure may benefit from immune therapy (Clark et al., 2006). Other options, such as prednisolone, are currently under investigation by us (GS) and others (Tang et al., 2009). Immune therapy can be expensive and potentially harmful, and is not yet something that should be considered routine clinical practice. So it is essential, for the sake and safety of our patients, to continue seeking effective ways of determining which women should try immune therapy for recurrent reproductive failure. Endometrial biopsy assessment of immune cells, performed carefully and in a systematic way, is one possible screening option, but is unlikely to be the only factor in clinical decision-making.

References

Akdis, M., Simon, H.U., Weigl, L., Kreyden, O., Blaser, K., Akdis, C.A., 1999. Skin homing (cutaneous lymphocyte-associated antigen-positive) CD8+ T cells respond to superantigen and contribute to eosinophilia and IgE production in atopic dermatitis. J. Immunol. 163, 466–475.

Aoki, K., Kajiura, S., Matsumoto, Y., Ogasawara, M., Okada, S., Yagami, Y., Gleicher, N., 1995. Preconceptional natural-killer-cell activity as a predictor of miscarriage. Lancet 345, 1340–1342.

Bankfalvi, A., Boecker, W., Reiner, A., 2004. Comparison of automated and manual determination of HER2 status in breast cancer for diagnostic use: a comparative methodological study using the Ventana BenchMark automated staining system and manual tests. Int. J. Oncol. 25, 929–935.

Barash, A., Dekel, N., Fieldust, S., Segal, I., Schechtman, E., Granot, I., 2003. Local injury to the endometrium doubles the incidence of successful pregnancies in patients undergoing in vitro fertilization. Fertil. Steril. 79, 1317–1322.

Brinkmann, V., Kristofic, C., 1997. Massive production of Th2 cytokines by human CD4+ effector T cells transiently expressing the natural killer cell marker CD57/HNK1. Immunology 91, 541–547.

Bulmer, J.N., Lunny, D.P., Hagin, S.V., 1988. Immunohistochemical characterization of stromal leucocytes in nonpregnant human endometrium. Am. J. Reprod. Immunol. Microbiol. 17, 83–90.

Choudhury, S.R., Knapp, L.A., 2001. Human reproductive failure I: immunological factors. Hum. Reprod. Update 7, 113–134.

Christiansen, O.B., Nielsen, H.S., Kolte, A.M., 2006. Future directions of failed implantation and recurrent miscarriage research. Reprod. Biomed. Online 13, 71–83.

Clark, D.A., Coulam, C.B., Stricker, R.B., 2006. Is intravenous immunoglobulins (IVIG) efficacious in early pregnancy failure? A critical review and meta-analysis for patients who fail in vitro fertilization and embryo transfer (IVF). J. Assist. Reprod. Genet. 23, 1–13.

Clifford, K., Flanagan, A.M., Regan, L., 1999. Endometrial CD56+ natural killer cells in women with recurrent miscarriage: a histomorphometric study. Hum. Reprod. 14, 2727–2730.

Croy, B.A., Esadeg, S., Chantakru, S., Van Den Heuvel, M., Paffaro, V.A., He, H., Black, G.P., Ashkar, A.A., Kiso, Y., Zhang, J., 2003. Update on pathways regulating the activation of uterine Natural Killer cells, their interactions with decidual spiral arteries and homing of their precursors to the uterus. J. Reprod. Immunol. 59, 175–191.

Davey, F.R., Elghetany, M.T., Kurec, A.S., 1990. Immunophenotyping of hematologic neoplasms in paraffin-embedded tissue sections. Am. J. Clin. Pathol. 93, S17–S26.

Dawood, F., Farquharson, R., Quenby, S., Toh, C.H., 2003. Acquired activated protein C resistance may be a risk factor for recurrent fetal loss. Fertil. Steril. 80, 649–650.

Dhont, M., 2003. Recurrent miscarriage. Curr. Womens Health Rep. 3, 361–366.

Disep, B., Innes, B.A., Cochrane, H.R., Tijani, S., Bulmer, J.N., 2004. Immunohistochemical characterization of endometrial leucocytes in endometritis. Histopathology 45, 625–632.

Fukui, K., Yoshimoto, I., Matsubara, K., Hori, R., Ochi, H., Ito, M., 1999. Leukocyte function-associated antigen-1 expression on decidual natural killer cells in patients with early pregnancy loss. Mol. Hum. Reprod. 5, 1083–1088.

Hazan, A.D., Smith, S.D., Jones, R.L., Whittle, W., Lye, S.J., Dunk, C.E., 2010. Vascular-leukocyte interactions: mechanisms of human decidual spiral artery remodeling in vitro. Am. J. Pathol. 177, 1017–1030.

Hill, J.A., Choi, B.C., 2000. Maternal immunological aspects of pregnancy success and failure. J. Reprod. Fertil. Suppl. 55, 91–97.

Jones, R.K., Bulmer, J.N., Searle, R.F., 1998. Phenotypic and functional studies of leukocytes in human endometrium and endometriosis. Hum. Reprod. Update 4, 702–709.

King, A., Burrows, T., Verma, S., Hiby, S., Loke, Y.W., 1998. Human uterine lymphocytes. Hum. Reprod. Update 4, 480–485.

King, K., Smith, S., Chapman, M., Sacks, G., 2010. Detailed analysis of peripheral blood natural killer (NK) cells in women with recurrent miscarriage. Hum. Reprod. 25, 52–58.

Konno, R., Igarashi, T., Okamoto, S., Sato, S., Moriya, T., Sasano, H., Yajima, A., 1999. Apoptosis of human endometrium mediated by perforin and granzyme B of NK cells and cytotoxic T lymphocytes. Tohoku J. Exp. Med. 187, 149–155.

Lachapelle, M.H., Miron, P., Hemmings, R., Roy, D.C., 1996. Endometrial T, B, and NK cells in patients with recurrent spontaneous abortion. Altered profile and pregnancy outcome. J. Immunol. 156, 4027–4034.

Laird, S.M., Tuckerman, E.M., Cork, B.A., Linjawi, S., Blakemore, A.I., Li, T.C., 2003. A review of immune cells and molecules in women with recurrent miscarriage. Hum. Reprod. Update 9, 163–174.

Lobo, S.C., Huang, S.T., Germeyer, A., Dosiou, C., Vo, K.C., Tulac, S., Nayak, N.R., Giudice, L.C., 2004. The immune environment in human endometrium during the window of implantation. Am. J. Reprod. Immunol. 52, 244–251.

Lukassen, H.G., Joosten, I., Van Cranenbroek, B., Van Lierop, M.J., Bulten, J., Braat, D.D., Van Der Meer, A., 2004. Hormonal stimulation for IVF treatment positively affects the CD56bright/CD56dim NK cell ratio of the endometrium during the window of implantation. Mol. Hum. Reprod. 10, 513–520.

Madsen, M., Moller, H.J., Nielsen, M.J., Jacobsen, C., Graversen, J.H., Van Den Berg, T., Moestrup, S.K., 2004. Molecular characterization of the haptoglobin–hemoglobin receptor CD163. Ligand binding properties of the scavenger receptor cysteine-rich domain region. J. Biol. Chem. 279, 51561–51567.

Maruyama, T., Makino, T., Sugi, T., Matsubayashi, H., Ozawa, N., Nozawa, S., 1992. Flow-cytometric analysis of immune cell populations in human decidua from various types of first-trimester pregnancy. Hum. Immunol. 34, 212–218.

Michimata, T., Tsuda, H., Sakai, M., Fujimura, M., Nagata, K., Nakamura, M., Saito, S., 2002. Accumulation of CRTH2-positive T-helper 2 and T-cytotoxic 2 cells at implantation sites of human decidua in a prostaglandin D(2)-mediated manner. Mol. Hum. Reprod. 8, 181–187.

Michimata, T., Sakai, M., Miyazaki, S., Ogasawara, M.S., Suzumori, K., Aoki, K., Nagata, K., Saito, S., 2003. Decrease of T-helper 2 and T-cytotoxic 2 cells at implantation sites occurs in unexplained recurrent spontaneous abortion with normal chromosomal content. Hum. Reprod. 18, 1523–1528.

Moffett, A., Regan, L., Braude, P., 2004. Natural killer cells, miscarriage, and infertility. BMJ 329, 1283–1285.

Myers, E.R., Silva, S., Barnhart, K., Groben, P.A., Richardson, M.S., Robboy, S.J., Leppert, P., Coutifaris, C., 2004. Interobserver and intraobserver variability in the histological dating of the endometrium in fertile and infertile women. Fertil. Steril. 82, 1278–1282.

Nguyen, T.T., Schwartz, E.J., West, R.B., Warnke, R.A., Arber, D.A., Natkunam, Y., 2005. Expression of CD163 (hemoglobin scavenger receptor) in normal tissues, lymphomas, carcinomas, and sarcomas is largely restricted to the monocyte/macrophage lineage. Am. J. Surg. Pathol. 29, 617–624.

Norwitz, E.R., Schust, D.J., Fisher, S.J., 2001. Implantation and the survival of early pregnancy. N. Engl. J. Med. 345, 1400–1408.

Noyes, R.W., Hertig, A.T., Rock, J., 1975. Dating the endometrial biopsy. Am. J. Obstet. Gynecol. 122, 262–263.

Paidas, M.J., Ku, D.H., Davis, B., Lockwood, C.J., Arkel, Y.S., 2004. Soluble monocyte cluster domain 163, a new global marker of anti-inflammatory response, is elevated in the first trimester of pregnancy. J. Thromb. Haemost. 2, 1009–1010.

Pandey, M.K., Rani, R., Agrawal, S., 2005. An update in recurrent spontaneous abortion. Arch. Gynecol. Obstet. 272, 95–108.

Perez, S.A., Sotiropoulou, P.A., Gkika, D.G., Mahaira, L.G., Niarchos, D.K., Gritzapis, A.D., Kavalakis, Y.G., Antsaklis, A.I., Baxevanis, C.N., Papamichail, M., 2003. A novel myeloid-like NK cell progenitor in human umbilical cord blood. Blood 101, 3444–3450.

Petitbarat, M., Serazin, V., Dubanchet, S., Wayner, R., De Mazancourt, P., Chaouat, G., Ledee, N., 2010. Tumor necrosis factor-like weak inducer of apoptosis (TWEAK)/fibroblast growth factor inducible-14 might regulate the effects of interleukin 18 and 15 in the human endometrium. Fertil. Steril. 94, 1141–1143.

Quenby, S., Bates, M., Doig, T., Brewster, J., Lewis-Jones, D.I., Johnson, P.M., Vince, G., 1999. Pre-implantation endometrial leukocytes in women with recurrent miscarriage. Hum. Reprod. 14, 2386–2391.

Quenby, S., Kalumbi, C., Bates, M., Farquharson, R., Vince, G., 2005. Prednisolone reduces preconceptual endometrial natural killer cells in women with recurrent miscarriage. Fertil. Steril. 84, 980–984.

Rai, R., Sacks, G., Trew, G., 2005. Natural killer cells and reproductive failure—theory, practice and prejudice. Hum. Reprod. 20, 1123–1126.

Rey, E., Kahn, S.R., David, M., Shrier, I., 2003. Thrombophilic disorders and fetal loss: a meta-analysis. Lancet 361, 901–908.

Rinehart, J., 2007. Recurrent implantation failure: definition. J. Assist. Reprod. Genet. 24, 284–287.

Stockinger, H., Majdic, O., Knapp, W., 1996. 1995 Directory for the human leukocyte clusters of differentiation. Transfusion (Paris) 36, 268–285.

Tan, B.K., Vandekerckhove, P., Kennedy, R., Keay, S.D., 2005. Investigation and current management of recurrent IVF treatment failure in the UK. BJOG 112, 773–780.

Tang, A.W., Alfirevic, Z., Turner, M.A., Drury, J., Quenby, S., 2009. Prednisolone Trial: study protocol for a randomised controlled trial of prednisolone for women with idiopathic recurrent miscarriage and raised levels of uterine natural killer (uNK) cells in the endometrium. Trials 10, 102.

Tawfik, O., Venuti, S., Brown, S., Collins, J., 1996. Immunohistochemical characterization of leukocytic subpopulations in chronic endometritis. Infect. Dis. Obstet. Gynecol. 4, 287–293.

Tincani, A., Balestrieri, G., Danieli, E., Faden, D., Lojacono, A., Acaia, B., Trespidi, L., Ventura, D., Meroni, P.L., 2003. Pregnancy complications of the antiphospholipid syndrome. Autoimmunity 36, 27–32.

Tuckerman, E., Laird, S.M., Prakash, A., Li, T.C., 2007. Prognostic value of the measurement of uterine natural killer cells in the endometrium of women with recurrent miscarriage. Hum. Reprod. 22, 2208–2213.

Vakkila, J., Lotze, M.T., Riga, C., Jaffe, R., 2005. A basis for distinguishing cultured dendritic cells and macrophages in cytospins and fixed sections. Pediatr. Dev. Pathol. 8, 43–51.

Vassiliadou, N., Bulmer, J.N., 1996. Immunohistochemical evidence for increased numbers of 'classic' CD57+ natural killer cells in the endometrium of women suffering spontaneous early pregnancy loss. Hum. Reprod. 11, 1569–1574.

Wang, D., Fung, J.N., Tuo, Y., Hu, L., Chen, C., 2010. TWEAK/Fn14 promotes apoptosis of human endometrial cancer cells via caspase pathway. Cancer Lett. 294, 91–100.

Warren, H.S., 2000a. Cd56. J. Biol. Regul. Homeost. Agents 14, 317–321.

Warren, H.S., 2000b. Cd56. J. Biol. Regul. Homeost. Agents 14, 322–323.

Wicherek, L., Basta, P., Pitynski, K., Marianowski, P., Kijowski, J., Wiatr, J., Majka, M., 2009. The characterization of the subpopulation of suppressive B7H4(+) macrophages and the subpopulation of CD25(+) CD4(+) and FOXP3(+) regulatory T-cells in decidua during the secretory cycle phase, Arias Stella reaction, and spontaneous abortion—a preliminary report. Am. J. Reprod. Immunol. 61, 303–312.

Winger, E.E., 2007. CD57+ cells and recurrent spontaneous abortion. Am. J. Reprod. Immunol. 58, 311–314.

Wold, A.S., Arici, A., 2005. Natural killer cells and reproductive failure. Curr. Opin. Obstet. Gynecol. 17, 237–241.

Yamada, H., Morikawa, M., Kato, E.H., Shimada, S., Kobashi, G., Minakami, H., 2003. Pre-conceptional natural killer cell activity and percentage as predictors of biochemical pregnancy and spontaneous abortion with normal chromosome karyotype. Am. J. Reprod. Immunol. 50, 351–354.

Yeaman, G.R., Collins, J.E., Fanger, M.W., Wira, C.R., Lydyard, P.M., 2001. CD8+ T cells in human uterine endometrial lymphoid aggregates: evidence for accumulation of cells by trafficking. Immunology 102, 434–440.