Arch Gynecol Obstet (2010) 281:983–990
DOI 10.1007/s00404-009-1179-9

MATERNO-FETAL MEDICINE

Specific secretory phase endometrial leukocytes of women with two and more consecutive idiopathic abortions are not significantly different from healthy controls

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Received: 13 April 2009 / Accepted: 30 June 2009 / Published online: 15 July 2009

Abstract
Objective To analyze concentrations of endometrial leukocytes in patients with idiopathic-repeated abortions.
Materials and methods Biopsies of exactly dated secretory endometrium in 25 patients with idiopathic-repeated abortions and 10 control patients without a history of miscarriage were compared with respect to the concentrations of T-helper cells (CD4), cytotoxic T-cells (CD8), B-cells (CD19) and uterine natural killer cells (CD56) by immunohistochemistry and RNase protection assays.
Results All examined cells were detectable within secretory endometrium. No statistically significant differences of the examined immune-cell concentrations were seen between the control group and the repeated miscarriage group by either test.
Conclusion This study suggests that the concentrations of specific endometrial leukocytes in a non-pregnant cycle are not associated with repeated pregnancy loss. Thus, the hypothesis of an altered endometrial immunity in patients with repeated miscarriages, symbolized by persistently differing local immune-cell concentrations, has to be questioned.

Keywords Endometrium · Recurrent spontaneous abortions · Idiopathic · Leukocytes · mRNA · Protein

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Introduction

Recurrent spontaneous early abortions (RSA) are defined as the loss of at least three consecutive pregnancies before the end of the first trimester of gestation and affect up to 5% of fertile couples [1]. The risk of recurrence increases with the number of previous, successive miscarriages and with maternal age [2]. In recent years, however, patients older than 30 years with two consecutive miscarriages are proposed to be included in screening examinations for couples with repeated abortions as well [3, 4].

Genetic examination of tissue from recurrent abortions more often reveals normal embryonic karyotypes compared to sporadic abortions [5–9], suggesting a disturbed maternal–embryonic interaction as a possible reason for miscarriages.

A number of underlying pathologies have been attributed to the development of recurrent abortions, such as balanced parental chromosomal abnormalities, maternal endocrinopathies, acquired or hereditary thrombophilic disorders and different forms of uterine malformation [2, 10–12]. However, the etiology of recurrent abortions remains unclear in up to 50% of all cases, despite an intensive and expensive work-up [13, 14]. The reciprocal interaction between the embryo and endometrial cells is regarded as crucial for the further development of the pregnancy [15]. A disturbed maternal acceptance of the semi-allogenic embryo at the implantation site and the decidua has thus been attributed to recurrent miscarriages [10].

Therefore, endometrial samples during the "window of implantation" [16] in the secretory phase have been used as a model. Several authors examined leukocyte concentrations in patients with recurrent miscarriages [17–23] to find possible differences between RSA patients and healthy controls and even to predict the outcome of future

pregnancies [17]. However, most studies [17–19] lack sufficient exclusion of other possible reasons for abortion to classify the patients as idiopathic aborters, without identifiable causes of miscarriage in either partner. Thus, the role of endometrial immune cells in the development of recurrent idiopathic abortion and the hypothetic role of allo-immunologically mediated miscarriage still remain unclear.

To address the question of possibly altered endometrial immune-cell concentrations, we examined the concentrations of different maternal endometrial leukocytes by two different methods in exactly dated secretory endometrial tissue of healthy controls and patients with repeated, consecutive abortions after definite exclusion of all other established causes for recurrent miscarriages.

Materials and methods

Ethical approval according to the principles set out in the Declaration of Helsinki and written consent from each patient was obtained after the purpose of the study had been fully explained.

Patients

We used the database of our institution to identify women who had undergone the extensive screening program for RSA in our outpatient clinic and had been classified as idiopathic-repeated aborters before the 12th week of gestation according to standards published elsewhere [10]. In brief, maternal endocrinopathies [hyperprolactinemia, luteal insufficiency (detected due to repeatedly decreased luteal progesterone levels), hyper- and hypothyreosis, hyperandrogenemia, polycystic ovary syndrome or hypersecretion of the luteinizing hormone (LH) and insulin resistance], acquired (anti-phospholipid syndrome) or hereditary thrombophilic disorders [resistance against activated protein C due to a factor V Leiden mutation, Prothrombin (G20210A) mutation, deficiencies of coagulation factors XII or XIII, reduced activity of protein S, protein C and antithrombin, hyperhomocysteinemia, C677T polymorphisms of the methylenetetrahydrofolate reductase] and different forms of uterine malformation had been ruled out by blood tests or ultrasound and hysteroscopy. Furthermore, karyotype analysis had been performed before in all women and their partners, identifying those without chromosomal abnormalities. Patients with a history of proven embryonic causes for miscarriages, such as embryonic karyotype abnormalities (when available) in a previous pregnancy, were excluded from the study.

Collection of material

Thus, endometrial tissue from regularly cycling women with repeated spontaneous early abortions was collected in the luteal phase of the menstrual cycle; 8–9 days after a commercially available urine test (Clearplan, Unipart; Cologne, Germany) indicated the peak of the LH. Overall, 25 patients were included, 17 of those had suffered from three and more idiopathic early miscarriages, whereas eight women, older than 30 years, had a history of exactly two early abortions. Ten healthy women without a history of adverse pregnancy outcome served as control. None of the patients was hormonally stimulated. An interval of at least 3 months between the last pregnancy or a hormonal therapy and the collection of material was observed for both groups. Endometrial samples were collected transcervically through a small biopsy catheter (Suresample, SIMS Portex Ltd., Hythe, UK).

We chose CD4-positive (helper T-cells), CD8-positive (cytotoxic T-cells), CD19-positive (B-cells) and CD56-positive (endometrial granulated lymphocytes/uterine natural killer cells) cells as examples of different immune-competent leukocytes in the endometrium, whereas cells positive for the panleukocyte marker CD45, indicating the overall amount of endometrial leukocytes, served as positive control.

For immunohistochemistry and for RNase protection assays (RPAs), tissue samples of approximately 5 mm in diameter were snap frozen and stored at −80°C.

Beside the positive LH peak 8–9 days before, endometrial samples from each patient were dated according to the last menstrual period, the histology [24] and the serum levels of 17β-estradiol and progesterone at the time of sampling. For endometrial dating, 5-µm sections stained with hematoxylin and eosin were evaluated.

Immunohistochemical staining

Immunohistochemical staining was performed in duplicates as described elsewhere [25]. Frozen sections (10-µm thick) were stained using commercially available kits (Histostain-SP Kits, Zymed Laboratories, San Francisco, USA).

Sections were incubated with monoclonal mouse anti-human antibodies, directed against CD4, CD8, and CD19 (all from Dako, Glostrup, Denmark) and CD56 (Becton Dickinson San Jose, USA) at a concentration of 1:100. Negative controls included staining without the primary antibody, whereas positive controls were performed using a monoclonal mouse anti-human CD45 antibody (Dako, Glostrup, Denmark) at the same concentration as the primary antibody. In a second step, the sections were incubated with anti-mouse antibodies to enable staining with the avidin–biotin method as described before [25].

The intensity of the immunohistochemical staining was semi-quantitatively assessed independently by two investigators (M.K.B., M.v.W.). Using a score (0 = absent, 1 = weak, 2 = intermediate, or 3 = strong), the intensity of the staining was interpreted in accordance to the method published by Tawia et al. [26]. All slides were evaluated using a Leica microscope (Leica DMLB, Leica Bensheim, Germany); both investigators were blind with respect to the group from which the tissue sample originated.

RNase protection assay

We then confirmed subpopulations of the immunohistochemically studied subtypes of leukocytes by RNA isolation and RPA, performed according to a protocol published elsewhere [27]. In brief, following homogenization of the endometrial tissue, total RNA was isolated by Trizol (Life Technologies, Karlsruhe, Germany), based on phenol–chloroform extraction. RPAs were performed using the RiboQuant kit (Pharmingen, San Diego, USA). Probes and negative controls (yeast RNA) and positive controls were purchased from Pharmingen. Semi-quantification was achieved by normalizing the optical densities of the specific bands to the optical densities of the housekeeping genes, GAPDH and L32. The optical density of the specific protected bands was expressed as relative values. A twofold increase of the relative optical density values corresponded, on average, to a 1.8-fold increase of the specific RNA, as determined in several dilution series.

Statistics

Results of immunohistochemistry and RPA were compared between the two groups using the Mann–Whitney test for non-parametric data (GraphPad Prism 4.0 software, GraphPad Software, San Diego, CA). Significance was established at the $p < 0.05$ level. We hypothesized that due to the lack of other established risk factors for recurrent abortions an immunological cause for miscarriages might be present in the majority of patients with recurrent abortions. We thus assumed that 80% of the patients should have intermediate or strong presence of endometrial leukocytes according to a specific score in immunohistochemistry. According to our prior analysis (N-Query advisor, Statcon, Witzenhausen, Germany), 25 samples of patients and 10 controls were needed in order to achieve a power of 85% with an $\alpha = 0.05$ in order to detect a difference of 60% in staining.

Results

The patients' characteristics are shown in Table 1.

Table 1 Demographic data of the study groups, data are mean ± standard deviation, when applicable

	Controls	Miscarriages
Number of patients	10	25
Number of miscarriages	0	3.3 ± 1.17
Range	0	2–6
Age at examination	33.5 ± 4.3	32.8 ± 5.6
Range	23–37	21–41

Immunohistochemistry

Figure 1 shows representative staining results for the different scores. Immunohistochemistry confirmed the presence of all investigated endometrial leukocytes of secretory phase endometrium. Figure 2 shows representative staining results for CD45, CD4, CD8, CD19 and CD56. No significant difference between controls and patients with repeated abortions was observed by immunohistochemistry (Table 2).

Immune-cell antigen mRNA and protein expression in the endometrium

RNase protection assays of total endometrium confirmed expression of the examined leukocyte antigens and thus immune cells in the secretory phase in patients with repeated abortions and controls. No significant difference between controls and patients with repeated abortions was observed. The exact results are described in Table 3. Figure 3 shows a representative RPA. Due to the use of two different probe sets, the CD56 band was integrated into the figure at its corresponding nucleotide size.

Discussion

The leukocyte population accounts for more than 20% of the endometrial cell prior to implantation in the secretory phase [28]. The concentrations of different endometrial leukocytes have therefore been investigated in a number of studies to find links to the development of miscarriage [18–21]. However, as endometrial immune cells vary dramatically during the menstrual cycle [20, 28, 29], the exact timing of endometrial biopsy is vital to achieve comparable results. The best timing would be in early pregnancy, directly before the onset of another miscarriage. A number of research groups have thus examined endometrial tissue of patients with induced abortions as control groups and discovered differences to RSA patients after the onset of miscarriages. However, it remains unclear whether changes in the concentrations of decidual immune cells were cause

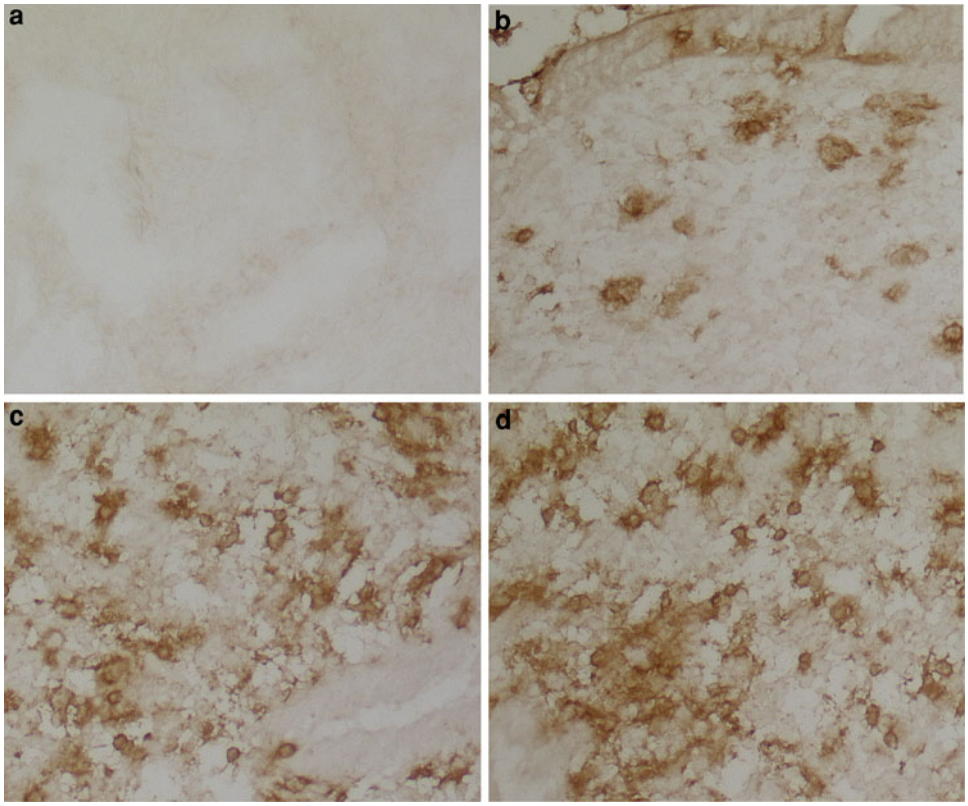

Fig. 1 Representative staining results for the different scores (antibodies against CD45 used in **b**–**d**; all micrographs ×400). **a** Intensity 0 = no staining (negative control); **b** intensity 1 = weak staining; **c** intensity 2 = intermediate staining; **d** intensity 3 = strong staining

or consequence of miscarriage, since endometrial biopsies in early pregnancy in patients with recurrent abortions would probably lead to adverse gestational outcome and are thus not performed.

Results from this study demonstrate that all investigated immune cells can be found in the endometrium during the secretory phase of patients with two and more consecutive abortions. No significant differences were observed between the examined patients and healthy controls without a history of miscarriage.

One main difference between this study and others is the fact that the biopsies were precisely timed with the timing confirmed by the combination of urinary test, histology and serum hormone levels. Furthermore, all other relevant established causes of recurrent miscarriages were ruled out, limiting our patients to those regarded as definitive idiopathic. This systematic exclusion of other possible causes for repeated miscarriages characterizes a major difference to the study of Quenby and co-workers [18] applying only the anti-phospholipid syndrome, maternal oligomenorrhea as a possible symptom of endocrinopathies and parental chromosomal anomalies as exclusion criteria. The application of two different examination methods—immunohistochemical staining and confirmation of the results by RPA—compared to only one method (immunohistochemistry) is a further difference to the publication of Quenby et al. Lachapelle et al. [17] excluded patients with thyroid dysfunction, hyperprolactinemia, specific auto-antibodies, infections, anatomical anomalies and parental chromosomal aberrations. However, their endometrial samples were dated between days 18 and 25 of the menstrual cycle, confirmed only by pathologic examination. With the number of endometrial leukocytes changing in correlation to the time after the LH peak [22], the publication of Lachapelle and co-workers may suffer from imprecise inclusion criteria. The study of Clifford and co-workers [19], showing an increased rate of endometrial CD56 positive natural killer cells in the luteal phase of women with idiopathic recurrent miscarriages, also lacked strict exclusion criteria, as only sonographically detected uterine anomalies, an anti-phospholipid syndrome and parental chromosomal anomalies served as exclusion criteria. As it is, e.g., well established that patients with thyroid dysfunction have an increased rate of miscarriage [30] and patients with autoimmune thyroiditis may have altered endometrial leukocyte concentrations [31], the lack of close-meshed exclusion criteria may thus lead to altered results in endometrial examination.

Since no concentration differences of the examined endometrial leukocytes between patients with repeated abortions in general and healthy controls could be observed in our study, the results of other groups [17–19] showing different immune-cell concentrations in a non-pregnant

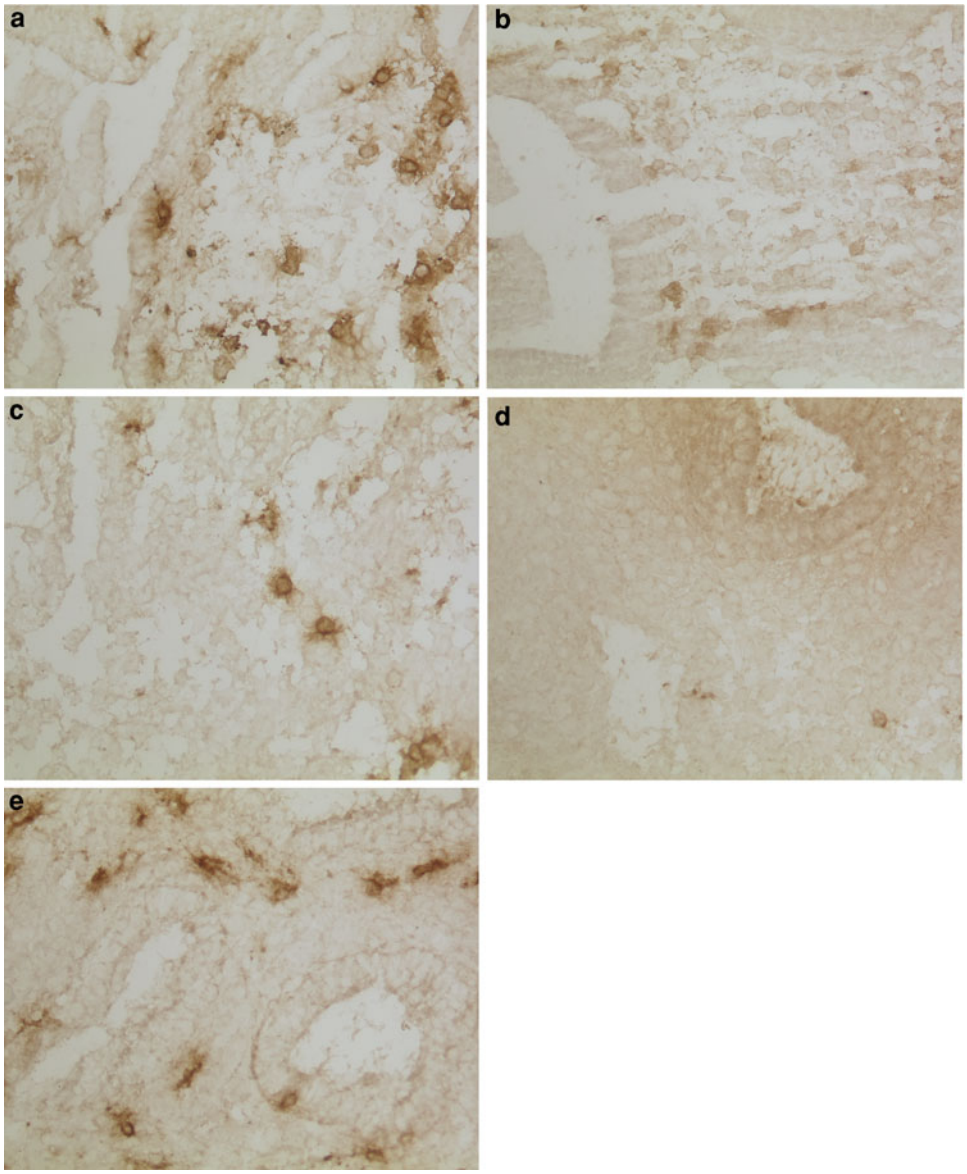

Fig. 2 Examples of immunohistochemical staining of endometrial leukocytes: **a** CD45 (positive control), **b** CD4, **c** CD8, **d** CD19, **e** CD56. All micrographs ×400

Table 2 The results of immunohistochemistry for endometrial leukocytes in the two study groups show non-significantly different *p* values

	Controls	Miscarriages	*p* value
CD4	1.80 ± 0.41	1.68 ± 0.60	0.24
CD8	1.89 ± 0.76	1.77 ± 0.60	0.65
CD19	0.38 ± 0.72	0.66 ± 0.64	0.10
CD56	2.78 ± 1.06	2.40 ± 0.62	0.88
CD45	2.71 ± 1.26	2.60 ± 0.96	0.42

Table 3 The results of the RNAse protection assays for endometrial leukocytes in the two study groups show non-significantly different *p* values

	Controls	Miscarriages	*p* value
CD4	44.16 ± 13.19	34.35 ± 13.81	0.12
CD8	20.06 ± 9.93	22.17 ± 9.34	0.68
CD19	59.80 ± 21.53	41.65 ± 17.03	0.05
CD56	4.63 ± 3.64	6.26 ± 5.09	0.76
CD45	16.12 ± 6.89	15.81 ± 9.11	0.19

Due to different assays and different band strengths results are only comparable within one line. The exact *p* value for CD19 was 0.053, thus not indicating a significant difference (as determined <0.05)

cycle leading to the hypothesis of a generally altered endometrial immune system in patients with repeated miscarriages have to be questioned. Our results are in line with the observations of Michimata et al. and Maruyama et al., who found no differences in endometrial leukocyte concentrations in patients with a history of exactly two abortions and healthy controls by means of immunohistochemistry [21] or differences in concentrations of CD19 and CD45 by flow cytometry [32].

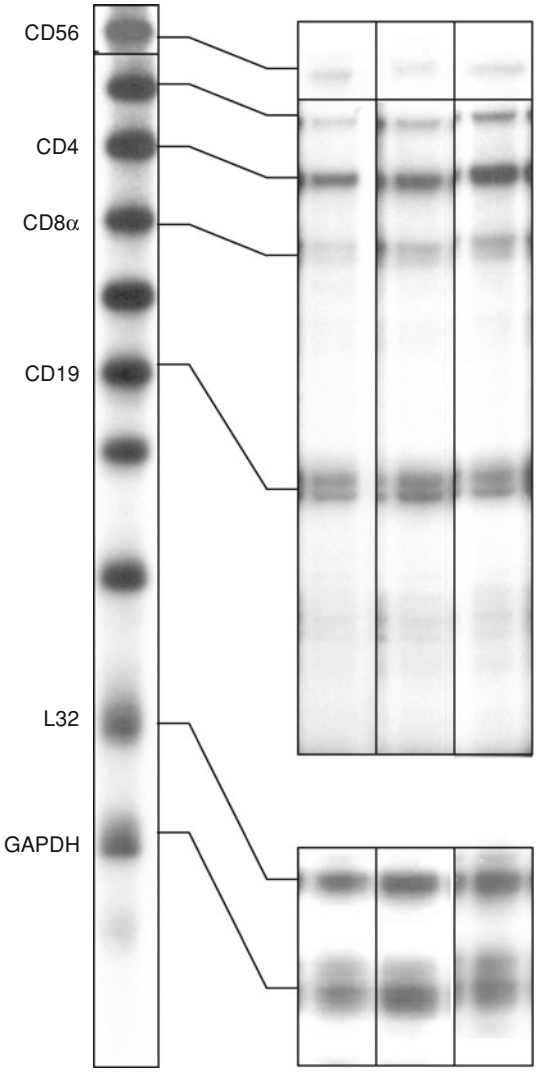

Fig. 3 Representative detail of the RNase protection assay. On the *left*, the unprotected probe with the respective mRNA sequences are shown, whereas on the *right*, probes from three study patients—one control and two with miscarriages—are displayed. The protected blots are matched to their corresponding unprotected probes. Due to the use of two different probe sets, the CD56 blot is integrated into the figure at its corresponding nucleotide size

The current study was designed to measure the concentrations of specific endometrial leukocytes. An abnormal expression of endometrial cytokines, e.g. interleukin (IL)-1beta and IL-6 mRNA [33], has been shown during the mid-secretory phase in patients with recurrent miscarriages. Local concentrations of IL-11 and leukemia inhibitory factor (LIF) have also been reported to be decreased in patients with recurrent miscarriages [34]. However, it has been criticized that the majority of studies have mainly concentrated on production of cytokines by endometrial immune cells, although they are also produced by local epithelial and stromal cells as well as the decidual and cytotrophoblast cells of the placenta [34]. In our study, the questions of different of endometrial cytokine concentrations [33–35] or protein expression [36, 37] were not addressed. It can therefore not be concluded from our results that functional immunological differences are not present in patients with repeated miscarriages compared to healthy controls.

One major limitation of examinations of endometrial tissue in a non-pregnant cycle is the fact that the different forms of interaction between maternal tissue and the invading trophoblast are not studied. However, the diagnostic dilemma in patients with otherwise idiopathic recurrent miscarriages remains that the measurement of peripheral immune cells is not a reliable parameter [38–41]. The concentrations of maternal immune cells in peripheral blood—suspecting a general "over-activation" of the maternal immune system—have been studied in recurrent aborters, but without unitary results. In recent years, special focus has been laid on the relationship of RSA and the number and activity of maternal natural killer (NK) cells. However, beside phenotypic and functional differences between peripheral and uterine NK cells [18, 42, 43], neither their concentration [38] nor their activity [44] in peripheral blood necessarily correlates with that of uterine NK cells. Furthermore, peripheral NK cells do not reflect a state of permanent over-active uterine immunity, since they may be transiently elevated due to blood withdrawal [45], stress, or due to ethnicity and age [2], whereas other factors, such as an elevated body-mass-index, do not seem to have an impact on endometrial leukocyte concentrations [46]. Therefore, several authors suggest not to draw conclusions from the measurement of peripheral immune cells, such as NK cells [38–41]. Thus, the analysis of biopsies is regarded as the gold standard of endometrial function, although the variability of endometrial development in different menstrual cycles can reduce its reliability [15, 36]. Thus, a major question remains whether an observed abnormality persists in subsequent cycles [47]. Furthermore, since invasive examinations of intact pregnancies in patients with recurrent abortions cannot be carried out [36], it remains speculative to assume that presumed immunological changes in the decidua occur prior to a subsequent miscarriage in repeated aborters. Comparing decidual leukocytes after such a subsequent miscarriage to findings in patients with elective pregnancy terminations of the same gestational age—as reported by other groups—may also lead to ambiguous results as hypothetical immunological differences might rather be the consequence than the cause of miscarriage. Although the concentrations of uterine immune-competent cells vary in chromosomally normal and abnormal miscarriages [48], some examinations lack karyotype analysis of the embryonic tissue, not excluding miscarriages due to embryonic aneuploidy [20].

Moreover, since only very little is known about the treatment of hypothetical endometrial defects [47, 49, 50] and

preventive approaches with active or passive immunization in patients with recurrent abortions are controversial, have not been successful and are generally not recommended [51, 52], no specific therapeutic options are yet known. Common recommendations, therefore, include frequent supportive care during pregnancy [10, 53–55], especially in patients with idiopathic miscarriages.

In summary, we were unable to show differences of concentrations of endometrial leukocytes between healthy controls and patients with repeated abortions after definite exclusion of all other established causes for recurrent miscarriages.

Acknowledgments We would like to thank Ms. Julia Jauckus, who contributed substantially to the accurate performance of the experiments.

Conflict of interest statement We declare that we have no conflict of interest.

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