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SHORT COMMUNICATION

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Uterine natural killer cells in patients with idiopathic recurrent miscarriage

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Problem: Uterine natural killer (uNK) cells are major players during implantation and early pregnancy. The aim of our study was to analyze uNK cell concentration in the endometrium of idiopathic recurrent miscarriage (iRM) patients and fertile controls.

Method of study: Out of n=130 couples with ≥3 consecutive, clinical RM screened according to a standardized diagnostic protocol, n=58 patients with iRM were identified. Endometrial biopsies were investigated in patients and n=17 fertile women (controls) via immunohistochemistry.

Results: Compared to controls, the concentration of uNK cells was significantly higher in iRM patients (257±212 vs. 148±73 uNK cells/mm², *P*=.04). IRM patients showed a higher prevalence of >300 uNK cells/mm² than controls (34.5% vs. 5.9%, *P*=.02). In 88% of controls and 62% of iRM patients, uNK cells were detected within the range of 40-300/mm².

Conclusion: Idiopathic recurrent miscarriage patients showed higher uNK cell levels than controls supporting the possible impact of uNK cells in the pathophysiology of miscarriage. Our cutoff levels might help to select RM patients which may benefit from immunomodulatory treatment.

KEYWORDS
endometrium, miscarriage, NK cells, uterus

1 | INTRODUCTION

Recurrent miscarriage (RM) is a highly frustrating disorder with an incidence of 1%-3% during reproductive years. Besides several established risk factors including anatomic, chromosomal, endocrine and hemostatic changes, the involvement of the immune system is discussed controversially. So far, only screening for antiphospholipid syndrome is recommended in guidelines as part of immunologic diagnostic.[1,2]

Several studies described immune parameters that showed changes in RM patients as compared to controls. One of these immune parameters of highly scientific and clinical interest are uterine natural killer (uNK) cells which form a large portion of immune cells in the uterine cavity and are major players during implantation and early pregnancy.[3] However, the distribution during the menstrual cycle and the pathophysiological significance in (early) pregnancy are only partially understood. Preliminary evidence indicates that the concentration of uNK cells in women with RM is increased.[4,5] However, controversial debates are ongoing and a standardized diagnostic method is still not established.[6-8] Analysis of uNK cells can be performed by fluorescence-activated cell sorting (FACS) or immunohistochemistry.[6] Reliable reference levels of uNK cells, which account for their tremendous changes depending on the day of cycle, for both techniques have not been determined yet. The aim of our study is to analyze uNK cell concentration in the endometrium of idiopathic RM patients and fertile controls to establish possible cutoff values.

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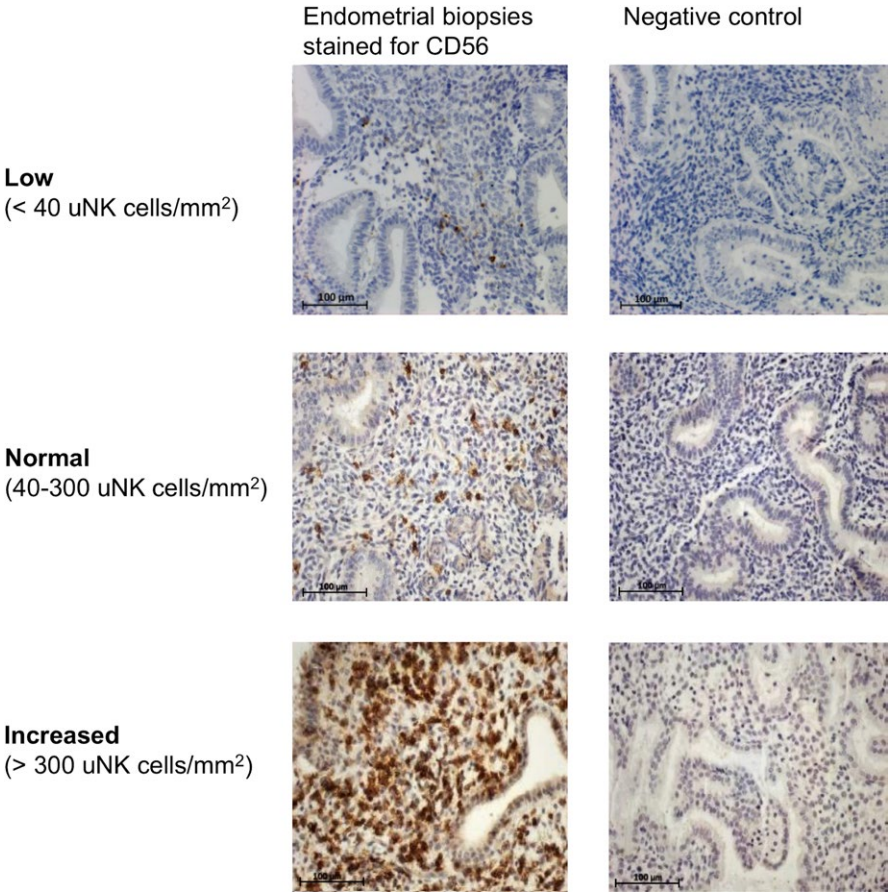

FIGURE 1 Endometrial samples stained for CD56 and negative controls. Immunohistochemical analysis of endometrial tissue for CD56+ uNK cells via DAB detection (brown signal). Score for semiquantitative evaluation: <40 uNK cells/mm²=low; 40-300 uNK cells/mm²=normal; >300 uNK cells/mm²=increased. Magnification ×200; scale bar 100 μm

2 | MATERIALS AND METHODS

2.1 | Study population

In total, n=130 patients, from 18 to 40 years of age, non-smoker with ≥3 consecutive, RM (confirmed as intrauterine miscarriages, by ultrasound or histology) were recruited between January 2014 and September 2016. Non-pregnant RM patients were routinely screened for anatomic disorders (vaginal ultrasound, office hysteroscopy), thyroid dysfunctions, anticardiolipin antibodies (IgG≥10 U/mL, IgM≥5 U/mL), anti-ß2-glycoprotein (IgG≥10 U/mL, IgM≥10 U/mL), lupus anticoagulant, deficiencies in coagulation factors (protein C, protein S, antithrombin), inherited thrombophilia (mutations in the factor V or prothrombin gene) and parental chromosomal disorders (numerical aberrations). In n=58 couples, idiopathic RM (iRM) was present (negative screening of the established risk factors). Control samples (n=17) have been obtained from egg donors of the Centro Médico Palencia Madrid, Spain. All egg donors recruited as controls had least one previous successful pregnancy and no autoimmune disease. N=3 controls showed a history of one early miscarriage. The Human Investigation Review Board at the Ruprecht-Karls University Heidelberg approved the study. All women included in this investigation gave their written consent.

2.2 | Detection of uterine natural killer cells

All biopsies were taken during the mid-luteal phase (7-10 days after LH surge) of a natural cycle.[9] The day of menstrual cycle has been confirmed by evaluation of endometrial glands and stroma. Endometrial biopsies were fixed in 5% buffered formalin for at least 24 hours and embedded in paraffin. The samples were cut at 4 μm, mounted on SuperFrost/Plus slides (Menzel, Germany) and deparaffinized and rehydrated. Antibodies were diluted with Background Reducing Components (DAKO, Germany). Antigen retrieval was accomplished by using citrate buffer. To inhibit endogenous peroxidase activity, samples were incubated with Peroxidase Block (DAKO, Germany) for 7 minutes as recommended and washed in TBS-Tween20 (0.05%; PBS, pH 7.6). Samples were incubated with the primary mouse antihuman CD56 antibody (clone:123C3, isotype: IgG1, DAKO, Germany) for 1 hour and for 30 minutes with the secondary antibody (labeled polymer-HRP antimouse, clone: DAK-GO1, isotype: IgG1, DAKO, Germany) at room temperature. Between each step, all samples were washed profusely with TBS-Tween20 (0.05%). The peroxidase reaction was achieved with DAB (3.3′-diaminobenzidine; DAKO, Germany) and discontinued with water after 15 minutes. Hematoxylin staining was followed by mounting the cover slide with Histofluid . All samples were analyzed independently by two experienced biologists/physicians using a Zeiss AxioPlan Microscope and the AxioVison 4.8

TABLE 1 Characteristics of iRM patients and healthy controls

	iRM≥3 (n=58)	Healthy controls (n=17)
Age[a]	34.7±3.6	27.0±9.0
Gravidity[b]	3 (3/14)	1 (1/2)
Parity[b]	0 (0/3)	1 (1/2)
No. of miscarriages[b]	3 (3/14)	0 (0/1)
GA of miscarriages[b]	8 (5/17)	n.a.
Time after miscarriage[b]	4 (2/16)	n.a.
BMI[a]	23.4±3.2	22.8±1.5

[a]mean±SD, [b]median (min/max). Characteristics: age (y), gravidity, parity, no. of miscarriages (number of miscarriages), GA of miscarriages (gestational age of miscarriages), time after miscarriage (time (months) to diagnostics after last miscarriage), BMI (body mass index); iRM ≥=patients with ≥3 idiopathic, consecutive, clinical miscarriages; healthy controls=egg donors with successful pregnancy and no autoimmune disease; n.a. =not assessed.

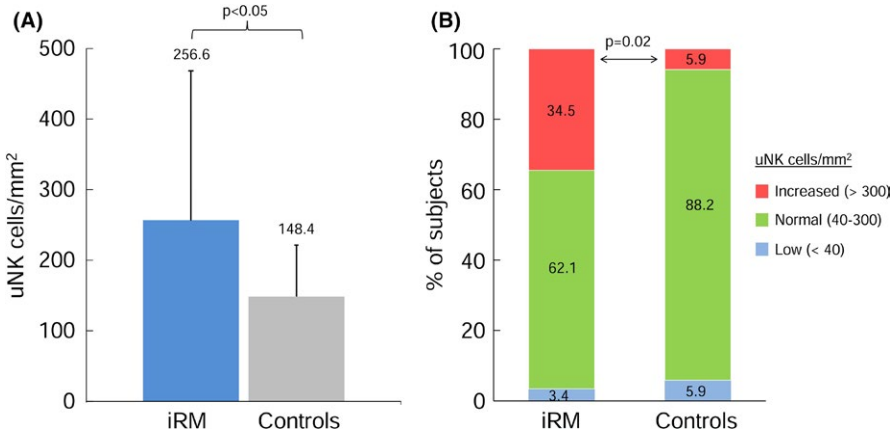

FIGURE 2 uNK cells in iRM patients and healthy controls. (A) Mean uNK cells/mm² in iRM patients are significantly higher compared to healthy controls. Student's *t* test was used, and a *P*-value<.05 was considered significant. Data are shown as mean+standard deviation. (B) Distribution of uNK cell counts in iRM patients and controls. The prevalence of >300 uNK cells/mm² is significantly higher in iRM patients compared to controls. Chi-square test was used, and a *P*-value<.05 was considered significant

program. CD56+ uNK cells were evaluated as absolute numbers per mm² (Figure 1).

2.3 | Statistics

Statistical analysis was performed using GraphPad Prism version 6.01 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. In case of normally distributed raw data, Student's *t* test was used to compare two groups. Data of dichotomous variables were compared by the Chi-square test. A *P*-value <.05 was considered significant.

3 | RESULTS

Characteristics of patients and controls are shown in Table 1. N=21 women of the RM group had a history of a livebirth prior to their miscarriages. In comparison with controls, endometrial biopsies of iRM patients contain significant higher levels of uNK cells (uNK cells/mm² in iRM patients: 257±212, controls: 148±73, *P*=.04, Figure 2A). About 88% of endometrial biopsies of controls showed a count of 40-300 uNK cells/mm². We suggest a score for the diagnostic evaluation of uNK cells/mm²: 0-40=low, 40-300=normal, >300 increased (uNK cells in controls: mean+2 standard deviations (SD): 295 uNK cells/mm²). Figure 2B shows the distribution of uNK cell counts in iRM patients and controls. The prevalence of samples >300 uNK cells/mm² is significantly higher in iRM patients compared to controls (34.5% vs. 5.9%, *P*=.02).

4 | DISCUSSION

Immunologic analyses are increasingly implemented in RM diagnostics. One of the parameters of interest are uNK cells. Our study reveals significant higher uNK cells in a strictly defined cohort of iRM patients compared to healthy control women.

First data indicating elevated uNK cells in iRM patients (n=20) compared to fertile controls (n=15) were provided by Lachapelle et al. in 1996.[10] In contrast to our study, uNK cells were identified by flow cytometry. The study described no significant difference in the overall number of uNK cells between women with RM and controls. However, in RM patients the rate of CD56+ cells that co-expressed CD16 was significantly higher, suggesting an important role of NK subsets in the pathophysiology of miscarriage.[10] In 1999, Clifford et al. published data on CD56+ uNK cells in n=29 women with ≥3 RM (normal parental chromosomes and uterine cavity in ultrasound scanning and negative for antiphospholipid antibodies) and n=10 parous women with no history of miscarriage or infertility.[4] Endometrial biopsies were taken during luteal phase and stained with anti-CD56. The morphometry was performed according to the "number of positively staining cells in 10 non-overlapping high-powered (X400) fields (h.p.f.) and repeated in a second specimen from the same subject and the mean of the absolute number of cells per 10 h.p.f. was calculated".[4] In line with our study, uNK cells were significantly higher in RM patients than in controls (mean±SD=146±71 per 10 h.p.f. in RM patients versus 94±19 per 10 h.p.f. in controls, *P*=.001).[4] Furthermore, RM patients with early

miscarriages showed even higher uNK cell counts (mean±SD=161±72 per 10 h.p.f., $P<.001$).[4] Quenby et al., again using immunohistochemistry, reported altered endometrial lymphocyte populations with elevated CD56+ uNK cells in endometrial biopsies from patients with ≥3 iRM (n=22).[5] Michimata et al. aimed to predict pregnancy outcome in n=17 iRM women (with two consecutive first-trimester miscarriages) as compared to n=15 women with male-factor infertility (controls).[11] RM patients and controls underwent endometrial biopsies during peri-implantation period.[11] In addition, subsequent pregnancy outcomes were documented. Immunohistochemistry was performed and CD56+ cells were counted per 10 h.p.f. like in the study of Clifford et al.[4] However, they did neither find significant differences between uNK cells in iRM patients and controls nor between patients with subsequent miscarriages or live births. Kamoi et al. investigated peripheral NK cells as well as uNK cells in women with ≥2 iRM (n=43) and unexplained infertility (n=38) by flow cytometry.[12] They were able to show that the percentage of endometrial CD56+/IL-22+ and CD56dim/IL-22+ cells in women with iRM was significantly higher than that of patients with unexplained infertility ($P<.05$).[12] A recent meta-analysis showed no significant difference of uNK cells between RM patients and controls.[6] In fact, we previously also reported no difference of uNK cells between various subgroups of RM (eg primary vs. secondary RM).[13] However, compared to healthy controls, this study shows significantly higher levels of uNK cells in a well-defined cohort of iRM patients. This highlights the importance to exclude established risk factors for RM and to use strict criteria of selection to identify new targets of diagnostics.

According to the mean value and standard deviation of controls, we suggest the following categories for uNK cell evaluation: 0-40 uNK cells/mm^2=low (5.9% of controls), 40-300 uNK cells/mm^2=normal (88.2% of controls) and >300 uNK cells/mm^2 (mean+2SD of controls 295 uNK cells/mm², 5.9% of controls). This concept needs to be confirmed in a larger control group, which is part of our ongoing research. Further, following up the obstetrical outcome of patients with low, normal and increased uNK cell levels is necessary to understand the impact of these cells on pregnancy outcome. It is important to prove our concept of reference categories for uNK cell evaluation with the help of clinical parameters (eg increased uNK cell levels are associated with a higher risk of miscarriage in a subsequent pregnancy).

Besides a standardized and strict protocol for assessing uNK cells by immunohistochemistry, it needs to be emphasized that timing is crucial to obtain reliable results of uNK cell diagnostics, as tremendous changes in uNK cell concentrations have been described for individual days of the menstrual cycle.[14]

5 | CONCLUSION

The endometrium of iRM patients contains higher concentrations of uNK cells as compared to controls. Future studies need to confirm reference values in a large cohort of control women. UNK cell diagnostics open a new target for immunomodulatory treatment strategies.

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