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Immune phenotype of the endometrium in patients with recurrent implantation failures after the transfer of genetically tested embryos in assisted reproductive technology programs

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ABSTRACT

Recurrent implantation failures (RIF) in assisted reproduction programs are one of the most challenging problems. Among the factors that can adversely affect implantation, endometrial immune structural disorders may be one of the leading causes. The aim of our work was to study the immune features of the endometrium in women with RIF after genetically tested embryo transfer in comparison with fertile gestational carriers. Immune cells in endometrial samples were studied by flow cytometry and RNA expression of IL (interleukin)15, IL18, fibroblast growth factor-inducible 14 receptor (Fn14), and tumor necrosis factor-like weak inducer of apoptosis (TWEAK) by reverse polymerase chain reaction. In one-third of the cases, a unique immune profile of the endometrium, which we called the not transformed endometrial immune phenotype, was found. It is characterized by a combination of features, such as high expression of HLA-DR on natural killers (NK), increased fraction of CD16 + , and a decreased fraction of CD56bright endometrial NK. In addition, when compared to gestational carriers, patients with RIF had a greater discrepancy between IL18 mRNA expression data, reduced mean TWEAK and Fn14 levels, and increased IL18/TWEAK and IL15/Fn14 ratios. Immune abnormalities that were found in more than half of the patients (66.7 %) may be the cause of implantation failures in genetically tested embryo transfer programs.

1. Introduction

Recurrent implantation failures (RIF) in assisted reproductive technology (ART) programs are among the most challenging problems in reproductive medicine. Even the transfer of chromosomally normal embryos does not result in live birth in 30 % of cases (Franasiak and Scott, 2017). Among the factors that can adversely affect implantation, endometrial immune structural disorders may be one of the leading causes (Kwak-Kim et al., 2021).

The decidualization of the endometrium occurs under the influence of ovarian hormones, mainly progesterone. It is characterized by the differentiation of endometrial stromal cells into specialized decidual cells, natural killers (NK) cell accumulation within the uterine mucosa, uterine vascular growth, and local edema (Gellersen and Brosens, 2014). The composition and function of many cells, including immune cells, change (Norwitz et al., 2001; Dunn et al., 2003; Kim et al., 2005; Arck and Hecher, 2013; Raghupathy and Szekeres-Bartho, 2022). Striking changes occur with uterine NK (uNK), which are considered "key" cells in implantation (Kwak-Kim and Gilman-Sachs, 2008; Huhn et al., 2021; Sacks and Finkelstein, 2021; Sfakianoudis et al., 2021; Zhang and Wei, 2021; Xie et al., 2022). In the follicular phase, NK account for approximately 10–20 % of all endometrial immune cells; after ovulation, their number is at least doubled and is already 40–50 % (King et al., 1989). These cells are mainly formed on the spot by differentiation of endometrial hematopoietic stem cells and tissue NK proliferation (Sojka et al., 2018), partially migrating from other places, including the

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peripheral blood (Manaster and Mandelboim, 2010; Gaynor and Colucci, 2017). Recruitment of NK cells to the endometrium, their proliferation (expansion), and maturation occur under the influence of interleukin (IL)15, which is produced by decidual stromal cells (Cooper et al., 2002; Blois et al., 2008; Manaster et al., 2008). Approximately 70–80 % of uNK cells are characterized as CD56brightCD16–. It is widely accepted that NK cells in the uterine mucosa are regulatory cells. Activated uNK cells produce different bioactive molecules: growth factors, angiogenic factors, cytokines. Another minor subpopulation of uNK cells, characterized as CD56dimCD16 + , displays cytotoxic activity (Giuliani et al., 2014). Decidual transformation is a dynamic process that begins after ovulation, during the secretory stage of the uterine cycle. It encompasses several steps: an acute pro-inflammatory initial secretory phase, which subsequently transitions to an anti-inflammatory secretory phase during embryo implantation. Endometrial NK cells target and eliminate senescent cells through granule exocytosis, primarily during the first pro-inflammatory stage. In addition, uNK cells produce a range of angiogenic mediators to provide peri-implantation growth and development of spiral arteries (branching, elongation, and vascular maturation), which increases the uterine vascular surface area in preparation for implantation during the anti-inflammatory secretory stage (Fraser and Zenclussen, 2022). One of the cytokines that regulates angiogenesis and remodeling of spiral vessels is IL18, which is produced by uNK and other endometrial cells (Petitbarat et al., 2011). Excessive levels of IL18 can shift the balance to the pro-inflammatory side and promote the conversion of uNK to cytotoxic cells (Petitbarat et al., 2011). Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) and its fibroblast growth factor-inducible 14 receptor (Fn14) play important roles in the regulation of cytokine balance, including IL18 levels (Burkly et al., 2011; Petitbarat et al., 2011).

Decidual NK have killer immunoglobulin-like receptors (KIRs) on the surface that recognize human leukocyte antigen (HLA)-C trophoblast molecules and are activated or inhibited, thus triggering a cascade of further interactions (Moffett et al., 2016). KIRs or CD158 molecules belong to the family of transmembrane proteins with either two (KIR2D) or three (KIR3D) immunoglobulin (Ig)-like extracellular domains. Some KIRs with long cytoplasmic domains contain tyrosine-based inhibitory motifs (ITIM) and possess inhibitory functions, while other KIRs with short cytoplasmic regions lack ITIM and have activating functions. NK cell activity depends on the balancing of signals between activatory and inhibitory receptors and the HLA-C type expressed by the trophoblast (Male et al., 2011; Moffett et al., 2016; Huhn et al., 2021; Xie et al., 2022).

For the last ten years, human endometrial immune cells have been the subject of a number of studies aimed at determining their levels at different phases of the cycle, including the receptivity period and the differences in these cell populations in healthy women and patients with RIF and other fertility disorders (Fukui et al., 2011; Junovich et al., 2013; Chernyshov et al., 2019; Marron and Harrity, 2019; Marron et al., 2019; Yang et al., 2019). Several markers of an impaired immune endometrial phenotype have been proposed, including an increased proportion of CD56dimCD16 + cells and CD56 + /-CD22 + cells in the mid-luteal endometrium (Fukui et al., 2011; Kamoi et al., 2015; Marron and Harrity, 2019; Marron et al., 2019). Several groups of researchers have studied the genome of the implantation window and receptive and non-receptive endometria (Koler et al., 2009; Díaz-Gimeno et al., 2011; Simon and Laufer, 2012; Díaz-Gimeno et al., 2013; Koot et al., 2016; Huang et al., 2017). Although the main purpose of these studies was to identify the optimal time for embryo transfer in ART programs, it is important that among the genes whose expression change during the implantation window and especially those ones that can predict implantation failure, a significant cluster of “immune” genes has been identified (Simon and Laufer, 2012; Ruiz-Alonso et al., 2013; Koot et al., 2016; Macklon, 2017). However, it was not possible to identify the immune causes of implantation failure in these studies. Therefore, many aspects of the human receptive endometrial immune phenotype remain unclear (Franasiak et al., 2021). Normal levels of various immune cells in the endometrium during the implantation window in the natural cycle and ART programs have not been clearly determined. It is unclear whether the different diagnostic techniques are comparable (Krjutškov et al., 2016; Yang et al., 2019). There are no proved diagnostic criteria that could be used in clinical practice (Shaulov et al., 2020; Mascarenhas et al., 2021; Günther et al., 2021). In terms of clinical application, the paper of Ledee et al. (2016), in which the authors studied the expression of IL15, IL18, TWEAK, and Fn14 mRNA by quantitative reverse transcription polymerase chain reaction (RT-PCR) and uNK by immunohistochemical method, identify different types of "immune profile" of the endometrium and offer different therapeutic approaches, seems to be promising.

Therefore, the objectives of our study were: 1) to compare immune cell populations, focusing on uNK, assessed by flow cytometry with IL18, IL15, TWEAK, and Fn14 mRNA expression in patients with RIF and fertile women (gestational carriers); and 2) to determine the presence, frequency, and nature of immune differences in the endometrium of patients with RIF in comparison with fertile women.

2. Material and methods

The study group (RIF – unexplained recurrent implantation failures) included 42 women with recurrent (at least three) consecutive implantation failures after the transfer of genetically (next generation sequencing) tested embryos aged 27–45 years, in 9 patients over 39 years old donor oocyte embryos were used. The duration of infertility in the RIF group was 9.4 ± 4.8 years, primary infertility occurred in 23 patients, and secondary infertility occurred in 19 patients. Five patients with RIF had 9 labors and 3 of them after ART. In 5 patients, 6 ectopic pregnancies happened, and 13 miscarriages occurred in 9 women (Table 1). At the initial examination of infertile couples, tubal-peritoneal infertility was detected in 16 women and chronic anovulation in 8 women, including 3 women with polycystic ovary syndrome and oligoasthenospermia in 3 men. Infertility of unknown origin was observed in eight couples.

All RIF patients were examined by 2D and 3D transvaginal ultrasound.

Hysteroscopy and immunohistochemical investigation of endometrial biopsy tissue for CD138 were performed in all patients with RIF before this investigation. At the time of the study, no patients had any signs of intrauterine pathology or chronic endometritis. None of the patients were positive for anticardiolipin antibodies, anti-beta-2-glycoprotein antibodies, and lupus anticoagulants. Levels of hormones, such as prolactin, free testosterone, and thyroid-stimulating hormone, were within normal ranges.

Women with uterine myoma, adenomyosis, endometriosis, uterine anomalies, thin endometrium, or intrauterine synechia were not included in this study. We also did not include patients with body mass index > 29 and those whose partners had severe oligoasthenospermia (less than 1 million motile sperm in the ejaculate).

Table 1
Clinical data of patients with RIF and GC.

Clinical data	RIF, n = 42	GC, n = 20
Age, years	37,3 ± 4,9 (from 27 to 45)	31,1 ± 4,7 (from 27 to 36)
Deliveries	9 deliveries in 5 patients (0,2±0,6)	49 deliveries in 20 women (2,5±2,5)
Miscarriages	13 missed abortions or early miscarriages in 9 women	6 miscarriages in 4 women
Extrauterine pregnancies	6 tubal pregnancies in 5 patients	0
Operations (caesarean section, laparoscopy, laparotomy)	5 laparoscopies, 1 laparotomy, 5 caesarean sections	1 laparoscopy

In total, in the past 194 transfers of 273 blastocyst stage embryos (including 190 genetically tested) were performed in the RIF group, 4.6± 2.5 transfers per patient on average, 6.5 ± 3.1 embryos, including 4.5 ± 1.1 euploid ones per patient.

The control group consisted of 20 gestational carriers (GC) aged 27–36 years. All women had at least one delivery. In total, there were 49 deliveries in this group. Five women had 9 medical abortions, and 4 women had 6 miscarriages. Nineteen women in the GC group underwent 40 transfers in ART programs, 50 embryos were transferred in general, 13 embryos were obtained after oocyte donation, and 30 embryos were genetically tested. There were 18 implantation failures in the GC group, which were not consecutive and not with genetically tested embryos.

All GC were examined using 2D and 3D transvaginal ultrasound. They were also tested for antiphospholipid antibodies and several hormones. All women of the GC group were negative for anticardiolipin antibodies, anti-beta-2-glycoprotein antibodies, and lupus anticoagulants. Prolactin, free testosterone, and thyroid-stimulating hormone levels were within normal ranges.

We realize that patients in our study and control groups were a priori not homogeneous by age and reproductive anamnesis. It is known that the main cause of implantation failure in older patients is embryo aneuploidy. As far as donor eggs were used in older patients (> 39 years), and all embryos were genetically tested, the age difference between our groups is obviously not essential. We mostly focused on the normal endometrium and uterus in both groups, confirmed implantation failure in patients with RIF, and proved successful implantation in the control group (GC).

The protocol of endometrial preparation for biopsy was identical to that used in the clinic for embryo transfer. Estrogens were administered from the 3rd day of the cycle: estradiol valerate (Proginova, Delfarm Lille, France for Schering AG, Germany) (2 mg) at a dose of 6 mg per day. On the 8th-11th day of estrogen administration, an ultrasound examination was performed to assess the thickness of the endometrium. When endometrial thickness reached at least 7 mm, dydrogesterone (Dufaston, Solvay, the Netherlands) (10 mg) was administered additionally at a daily dose of 60 mg. Endometrial biopsy was performed on the 7th-8th day of progesterone intake (P6-P7). Each woman's biopsy material (200–300 mm^3) was divided into several parts for the study of endometrial lymphocytes by flow cytometry and mRNA by quantitative RT-PCR, as well as for histological examination, which was not the subject of this study.

2.1. Endometrial lymphocytes study

The endometrial cell isolation method has been described previously (Chernyshov et al., 2019). Endometrial tissue samples were gently washed three times in phosphate-buffered saline (PBS). A 1 ml pipette tip was cut at the edge so that the diameter of the hole was 4 mm and endometrial tissue was washed 10–15 times by pipetting in cool (+4 to +9 °C) PBS in order to obtain free endometrial cells in PBS solution. The next pipette tip had a hole diameter of 2–3 mm and tissue pieces were pipetted again. In the last step of pipetting, the tip hole was 1–2 mm and pieces were pipetted without resistance, that is, every next step was performed with a smaller diameter of the tip hole. Between pipetting steps, the endometrium was washed with cold PBS by centrifugation. Endometrial tissue was disaggregated and flake-shaped. Seven milliliters of PBS (+4 °C) were added, mixed with the obtained suspension, and remained for 3–5 min for sedimentation of large aggregates. The cellular supernatant was carefully layered onto 3 ml of Histopaque-1077 in a 15 ml conical centrifuge tube and centrifuged at 400g for 30 min at room temperature. After centrifugation, the upper layer was carefully aspirated and the opaque interface was carefully transferred into a clean conical centrifuge tube. The cells were washed twice with 10 ml of PBS. Cells pellet was resuspended in 0.5 ml of PBS with 10 % of patient serum. The final cell suspension 80 μl was analyzed by three-color flow cytometry after staining with 5 μl monoclonal antibodies conjugated with fluorochromes FITC, PE, and PE-Cy 5 (BD Biosciences, USA) in the following combinations: CD158aFITC/CD56PE/CD3PECy5, CD8FITC/CD56PE/CD3PECy5, HLADRFITC/CD56PE/ CD3PECy5, HLADRFITC/CD8PE/CD3PECy5, HLADRFITC/CD4PE/CD3PECy5, CD16FITC/CD56PE/CD3PECy5, and CD3/CD335/CD56 (BD Biosciences, USA). Samples were vortexed for 30 s with 0.5 ml Lysing solution (BD Biosciences, USA) and washed with 10 ml of PBS. The samples were analyzed using a FACScan flow cytometer (BD Biosciences, USA). In addition, the samples were incubated with SimulTEST control (IgG1-FITC + IgG2-PE) as a background control. The gate strategy is shown in Supplementary Fig. 1.

2.2. Study of RNA expression

Biopsy fragments were fixed in RNAlater™ Stabilization Solution (Thermo Fisher Scientific, AM7021) to protect and stabilize RNA, rinsed with 1x PBS solution, and placed in a tube with a 10-fold volume of RNAlater solution added in accordance with the biopsy volume. The sample was stored at +4 °C for 24 h, and then the liquid excess was collected and disposed. The tube content remnants (samples) were placed in a freezer at −20 °C for long-term storage. For RNA isolation, the samples were pre-homogenized in liquid nitrogen. For RNA isolation, the NucleoSpin RNA kit, Mini kit (Macherey-Nagel, REF 740955.50) was used, and reverse transcription of RNA into cDNA was performed using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, K1621) according to the manufacturer's instructions.

Gene expression was studied using commercial TaqMan Gene Expression Assays for IL-15, IL-18, Fn14, TWEAK, and B2M (housekeeping) and PCR mix TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, 4305719) according to the manufacturer's recommended protocols. A QuantStudio™ 5 Real-Time PCR System (Applied Biosystems™, A34322) was used for amplification. The gene of interest expression index was calculated by the method of relative quantification and normalized to endogenous control (housekeeping) gene expression.

2.3. Statistical analysis

Statistical analysis was performed using the InStat software for Windows (version 3.0, Graph Pad Software Inc., San Diego, CA, USA). Significance of differences was calculated by the Mann-Whitney test (odd, nonparametric, bilateral) with 95 % confidence interval (CI). Pearson's and Spearman's correlation coefficients were used. Differences with $p < 0.05$ were considered statistically significant.

The Ethics Committee of Nadiya Clinic, where the study was planned, reviewed the proposed study protocol, agreed and approved it (Protocol № 3–18 of the meeting of Ethics Committee at LLC "Clinic of Reproductive Medicine "Nadiya"" from January 26, 2018). All patients and gestational carriers were informed about all stages of the study and provided written informed consent to participate.

3. Results

3.1. Lymphocytes populations in the endometrium of patients with RIF and GC

A comparison of lymphocyte subpopulations in the endometrium of patients with RIF and GC is presented in Table 2.

The mean numbers of uNK cells did not differ between the RIF and GC groups. A considerable decrease in uNK fraction among all lymphocytes (less than 20 %) was found only in one RIF patient (2.3 %), increased proportion of uNK (>65 %) - in seven (16.6 %) women with RIF. There were no such deviations in GC, but this difference between groups was not significant. However, the NK cell population in the group of patients with RIF was significantly different.

CD16, which is typical for blood and non-typical for endometrial NK,

Table 2
T lymphocytes and NK (%) in the endometrium of fertile women – GC and patients with RIF.

Groups and subgroups of T lymphocytes and NK cells (%)	GC, n = 17 Mean±SD	RIF, n = 42 Mean±SD
CD3 + (T cells)	48,8± 10,8	40,0* ± 13,4
HLA DR+ in T cells	48,0± 15,7	51,5 ± 20,7
CD158a+ in T cells	6,0± 11,9	24,7* ± 25,9
CD16 in CD3 +	1,9± 2,3	12,3* ± 19,9
CD3 +CD4 + (T helper cells)	14,3± 5,7	15,5 ± 7,9
HLA DR+ in T helper cells	28,7± 11,8	41,7* ± 18,3
CD3 +CD8 + (T cytotoxic cells)	31,6± 8,7	22,8* ± 8,5
HLA DR+ in T cytotoxic cells	55,3± 13,9	54,2 ± 20,9
CD56 + in T cytotoxic cells	18,4± 14,9	27,4 ± 22,7
CD4/CD8 ratio	0,5± 0,2	0,7* ± 0,4
CD56 +CD3- (NK cells)	34,7± 12,0	39,3 ± 15,3
CD158a+ in NK cells	19,3± 14,3	37,7* ± 21,7
HLA DR+ in NK cells	10,6± 6,3	21,4* ± 19,9
CD8+ in NK cells	32,4± 15,8	36,8 ± 18,5
CD56 + + bright in NK cells	66,5± 18,8	70,2 ± 16,4
CD16 + in NK	9,5± 9,9	22,8* ± 19,3
CD16 + in NK CD56 + +	5,2± 5,3	17,8* ± 17,6
CD16 + in NK CD56 +	20,3± 17,6	34,6* ± 22,5
NK CD335 +	94,0± 3,6	92,5 ± 10,2

* $p < 0.05$ compared to GC group.
The endometrial immune phenotype differs significantly between fertile women and patients with RIF. Immune peculiarities of the endometrium (significant differences in at least one parameter compared to GC) were found in 28 (66.7 %) RIF patients.

was more frequently expressed in NK cells of patients with RIF. More than 23 % of CD16 positive uNK cells were observed in 15 (35.7 %) women among 42 RIF patients and in one case (1 of 16 women) (6.2 %) in GC ($p = 0.0454$, OR=8.036, CI 95 %: 0.9648–66.926) (Fig. 1D). CD16 expression was increased in both CD56dim and CD56 bright subpopulations of NK cells.

Increased levels of HLA-DR positive uNK (more than 20 %) and increased HLA-DR expression on T helpers (Th) (more than 50 %) were observed in one woman from GC (1 of 17 women) (5.8 %) and in 17 of 42 (40.5 %) RIF patients ($p = 0.0113$, OR=10.880) (Fig. 1A, B). High expression of CD158a (more than 55 %) on uNK was found only in 1 of 17 GC women (5.8 %) versus 13 of 42 (30.9 %) RIF patients ($p = 0.0479$, OR=7.172) (Fig. 1C).

Quite often (13 patients, 30 %), such signs as an increase in the percentage of HLA-DR positive NK and Th, CD16 positive NK, and a decrease in the proportion of CD56bright NK were observed in the same patients.

Patients with RIF had a lower average number of T cells due to T cytotoxic lymphocytes (Tc) that led to a significant shift of Th/Tc ratio towards Th. The level of CD158a expression was more prominent on T cells. In addition, HLA-DR expression on Th was higher in patients with RIF (Fig. 1A). In the group of patients with RIF higher levels of NKT-like cells (CD3 +CD158a+, CD3 +CD16 +) were determined.

3.2. IL15, IL18, TWEAK, Fn14 mRNA expression in endometrial tissue of patients with RIF and GC

Data on mRNA expression in the endometrial tissues of the studied groups of women are presented in Fig. 2.

cU - conventional units of direct mRNA concentration (1/amplification cycle*10); * - $p < 0.05$; ns - The difference is insignificant.

The mean levels of IL15 and IL18 mRNA expression did not differ between the RIF and GC groups. TWEAK and Fn14 mRNA expression were lower and IL18/TWEAK and IL15/Fn14 ratios were higher in RIF patients than in GC women. Decreased levels of Fn14 and TWEAK mRNA expression were found in 18 (48 %) and 13 (35.1 %) of 37 study group cases correspondingly and were significantly associated with RIF (OR=14.211, $p = 0.0041$; OR=8.125, $p = 0.0410$) (Fig. 3). It was found that in GC, the distribution of IL18 mRNA expression data was denser, while in patients with RIF, there was both an increase and decrease (accentuations) in gene expression (Fig. 3). Increased IL18 mRNA expression was found in 13 (35.1 %) of 37 RIF patients compared to only 1 (6.2 %) case in 16 GC women (OR=8.125, $p = 0.0410$). The level of IL18 mRNA was reduced in 11 (29.7 %) of 37 cases compared to only 1 (6.2 %) case of 16 women in GC group (OR=6.346, $p = 0.0805$, which was not quite significant) (Fig. 3). Thus, the presence of accentuations in IL18 mRNA levels was highly correlated with RIF and was registered in the majority of patients (24 of 37 cases) (64.8 %) and in only 2 of 16 women in the GC group (12.4 %) (OR=12.923, $p = 0.0007$; OR=12.923, $p = 0.0007$).

3.3. Comparison of flow cytometry and gene expression data of the endometrium in patients with RIF and GC

When comparing gene expression in patients with immune accentuations detected by flow cytometry, it was found that there was a significant correlation between high HLA-DR levels in NK and T cells and decreased IL18 expression (Fig. 4). A similar negative correlation was observed between the expression of CD16 on NK and T cells and IL18 expression (Fig. 5). Thirteen patients (30 %) with high percentage of HLA-DR positive NK and T lymphocytes, CD16 positive NK, and low proportion of CD56bright NK had also decreased TWEAK and IL18 mRNA expression.

4. Discussion

In our study, we compared the endometrial immune phenotype in women with repeatedly failed (RIF) and proved successful (GC) embryo implantation. It was found that in a significant proportion of RIF patients (66.7 %), the endometrial immune phenotype was different from that in fertile women (GC).

There are no confirmed normal values for different subpopulations of endometrial lymphocytes at the moment. We obtained the reference values on the group of gestational carriers (this article) and egg donors from our previous data (Chernyshov et al., 2019). We considered the normal values to be within 90CI in fertile women (egg donors, gestational carriers).

4.1. Increased level of CD56 +CD16 + cells

Our results are partly in line with those of previous studies (Tuckerman et al., 2010; Santillán et al., 2015; Chen et al., 2017; Junovich et al., 2013; Marron and Harrity, 2019). These authors examined the immune cells of the middle secretory endometrium in patients with poor reproductive outcomes (RIF, recurrent miscarriages, and infertility). They found an increased number of blood-type NK, i.e., CD16 positive cells in patients with RIF. In our investigation, patients with RIF also had increased levels of CD56 +CD16 + cells (blood-type cells) and a decreased percentage of CD56 + +CD16-cells (uterine-type NK cells). CD16 cell surface receptors on NK cells activate antibody-dependent cell-mediated cytotoxicity and are important for the innate immune response. Uterine NK cells have reduced cytotoxicity levels compared to peripheral blood NK cells, and typically express little or no CD16 receptors. However, they can change their function and become more killer-like in the face of stress or infection (Sharma, 2014; Huber et al., 2021), thus creating an unfavorable environment for embryo implantation. Therefore, CD16 could be a promising marker of immune

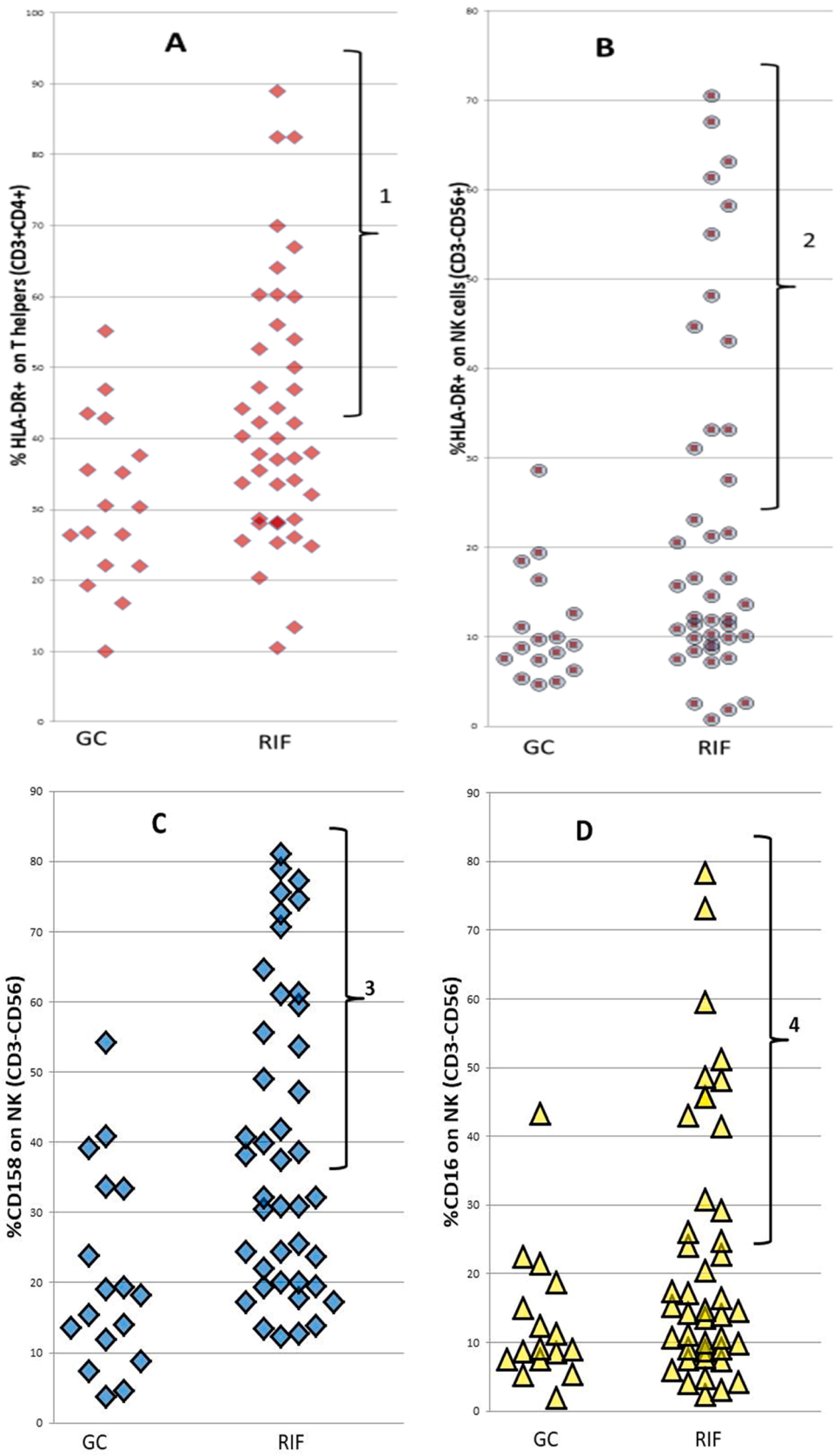


Fig. 1. Expression of HLA-DR on Th (A) and HLA-DR, CD158a and CD16 on endometrial NK (B, C, D) in GC group and in patients with RIF. Significantly associated with RIF (1 * OR-5.1 p = 0.0368 2 * OR-10.88 p = 0.0113 3 * OR-4.66 p = 0.0388 4 * OR-8.04 p = 0.0388).

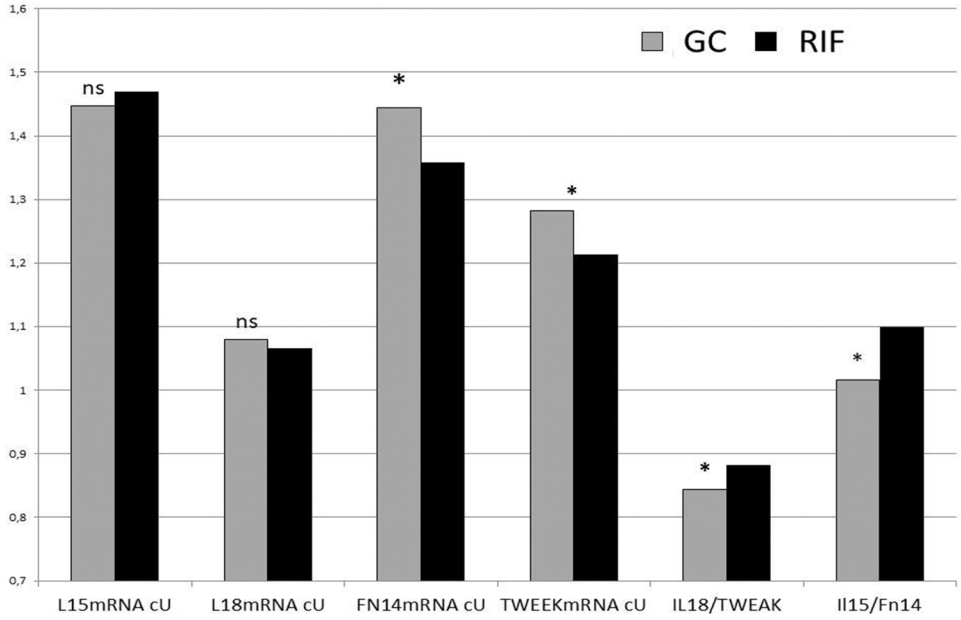


Fig. 2. RNA expression of IL15, IL18, TWEAK, Fn14 and IL18/TWEAK, IL15/Fn14 ratios in patients with RIF and GC.

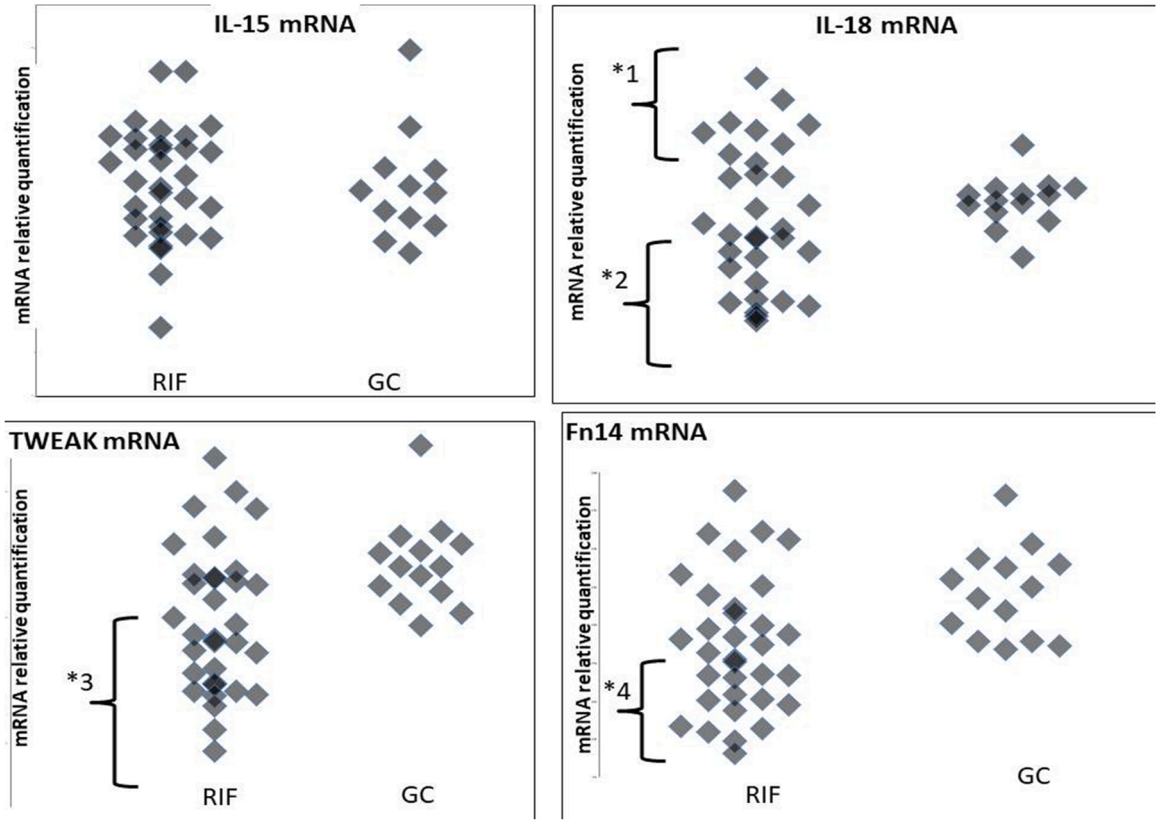


Fig. 3. Individual distribution of mRNA levels in the endometrium of RIF patients and GC. Significantly associated with RIF (1 * OR-8.1 p = 0.0410. 2 * OR-6.3 p = 0.0805. 3 * OR-8.1 p = 0.0410. 4 * OR-14.2 p = 0.0041).

abnormalities in idiopathic RIF (Huber et al., 2021).

4.2. Increase of HLA-DR expressing NK

In the available literature, we have not encountered information about the number and role of HLA-DR-positive uNK in the endometrium of healthy women, as well as in patients with infertility and RIF in ART programs. Unlike class I proteins of the major histocompatibility complex (MHC), which are expressed by most human cells, MHC class II proteins are mainly expressed by antigen-presenting cells. NK cells also express HLA-DR which is considered by some researchers as an activation marker; it appears on peripheral NK cells due to stimulation with IL2 (Evans et al., 2011). HLA-DR-positive NK cells in peripheral blood have been shown to capture and process certain antigens (tetanus toxin

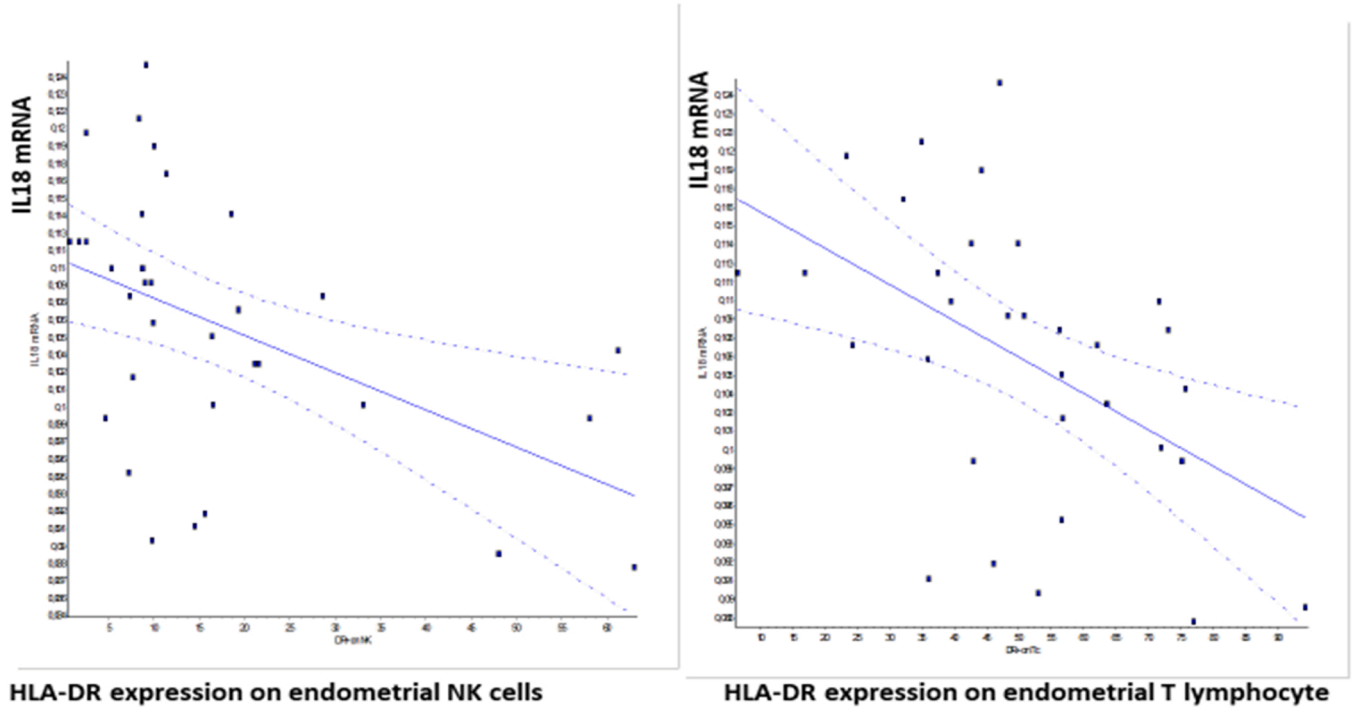


Fig. 4. Correlation between IL18 mRNA expression and HLA-DR levels on NK cells and T lymphocytes. Correlation for NK cells (r) = −0.482 p = 0.004 and T lymphocytes (r) = - 0.499 p = 0.003.

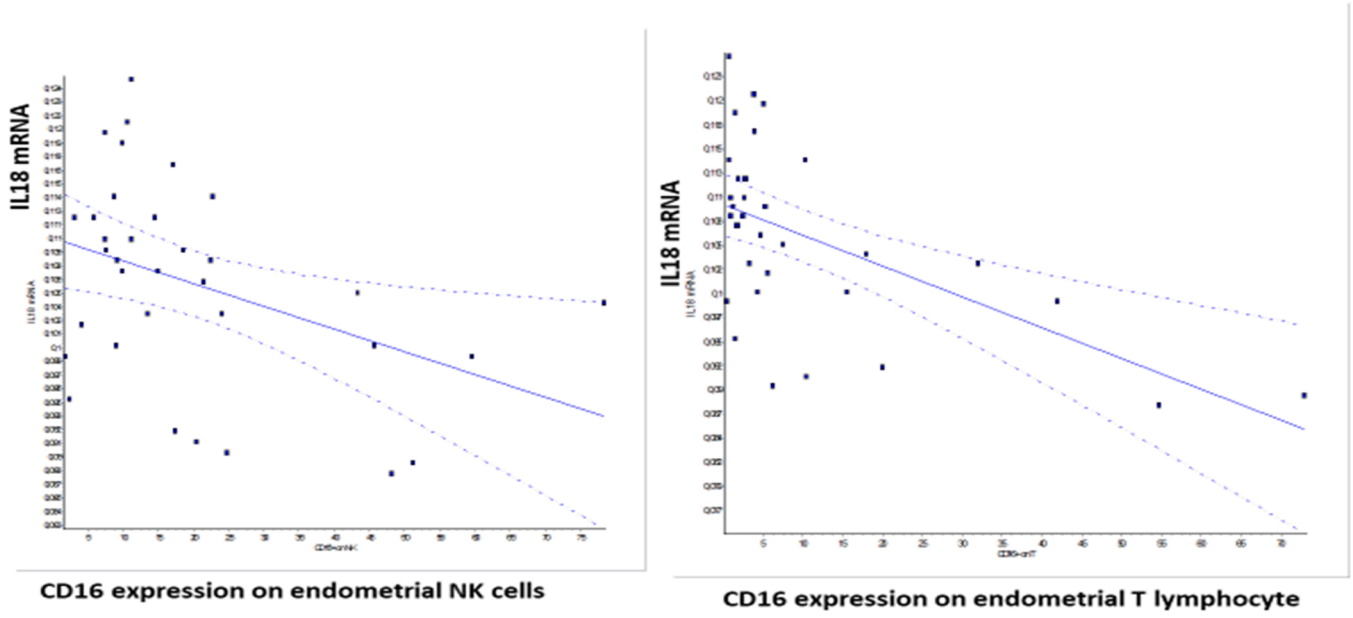


Fig. 5. Correlation between IL18 mRNA expression and CD16 levels on NK cells and T lymphocytes. Correlation for NK cells (r) = −0.403 p = 0.017 and T lymphocytes (r) = −0.567 p = 0.0005.

and house dust mite-derived peptides) for further HLA-DR-mediated presentation leading to T cell proliferation (Hanna et al., 2004). Early studies showed a correlation between the percentage of NK cells expressing HLA-DR and the level of interferon-gamma (IFN-γ) transcripts (Yano et al., 1996). NK cells that express HLA-DR have been found to have greater cytolytic activity and express chemokine receptors, which are associated with the recruitment of immune cells to sites of inflammation and lymph nodes. The number of HLA-DR-expressing NK cells in places of inflammation correlates with the severity of the immune disease (e.g., IgA nephropathy) (Yano et al., 1996). In addition, an increase in HLA-DR-positive NK cells in regional lymph nodes was detected in inflammatory conditions, such as chronic tonsillitis, and HLA-DR-positive decidual NK cells were found in cytomegalovirus-infected women (Hanna et al., 2004). HLA-DR-positive NK cells are constantly present in small amounts in peripheral blood and in greater numbers in the spleen, liver, lymph nodes, and other immunocompetent organs in healthy people. In the presence of infection, autoimmune disease, and oncological disease, the number of HLA-DR-positive NK cells increases because of direct exposure to infected cells, pathogens, or the action of pro-inflammatory cytokines.

The expansion of these cells is observed both in the peripheral blood and in the affected organs. HLA-DR-positive NK cells seem to be involved in first-line protection and in crosstalk with the adaptive immune system; they produce IFNγ, help exacerbate inflammation, can be cytotoxic, and trigger T cell activation. An expansion of HLA-DR-positive NK cells in the blood and tissues can persist for a long time, as long as inflammation exists, and then the number of these cells also decreases (Erokhina et al., 2021). Thus, it can be assumed that an increase in the number of HLA-DR-positive uNK cells in women with RIF may indicate a pro-inflammatory, ready to "fight the threat" phenotype. In our opinion, such a phenotype may be normal for the day of egg collection in the ART program (Chernyshov et al., 2019), perhaps for the day of ovulation in the natural cycle, but it is not perfect for the time of implantation window. Partly this endometrial lymphocyte phenotype resembled the immune accentuations of peripheral blood lymphocytes that we described earlier in patients with adverse prognosis of implantation or pregnancy course (Dons'koi et al., 2013; Dons'koi, 2015; Dons'koi et al., 2014; Dons'koi et al., 2016; Dons'koi et al., 2021), as well as in latent viral infection (Dons'koi et al., 2020). It appears that the expression of HLA-DR is a signal of alarm and danger. It was found that in patients with preeclampsia, the expression of HLA-DR is detected in microparticles from the placenta and that this is associated with aberrant syncytiotrophoblast surface expression in the chorionic villi (Tersigni et al., 2018). Typically, HLA-DR is silenced in syncytiotrophoblast. It is currently unclear whether HLA-DR from placental microparticles is of maternal or fetal origin. It is possible that placental cells receive HLA-DR from the decidua via trogocytosis (Tersigni et al., 2018). Thus, HLA-DR-positive uNK and T cells may be its source.

In a previous study, we showed that there are associations between CD8 levels in NK cells and HLA-DR levels in T lymphocytes and NK cells between peripheral blood lymphocyte phenotypes and endometrial lymphocytes (Chernyshov et al., 2019). The endometrium has a unique immune phenotype that is different from that of the peripheral blood, but it is not isolated from systemic accentuations of the immune system.

It turned out that three signs, such as an increased percentage of HLA-DR-positive NK, CD16 positive NK, and decreased proportion of CD56bright NK, often occurred in the same patients. Our previous investigation of oocyte donor endometrium in cycles of controlled ovarian stimulation showed that a similar immune structure was found on the day of egg collection, but did not occur on days corresponding to the implantation window (Chernyshov et al., 2019). In the present study, we also found no women with such high percentages of CD56 + HLA-DR+ and CD56 +CD16 + cells and a low proportion of CD56 + + cells in the GC group. As we know, the decidual transformation is not static, but a dynamic process. The initial pro-inflammatory response is crucial for the induction of endometrial receptivity; it is accompanied by a surge in free radical production and secretion of various cytokines, growth factors, and angiogenic factors (Fraser and Zenclussen, 2022). During this pro-inflammatory phase, uNK cells are involved in the targeting and elimination of senescent stromal cells (Strunz et al., 2021; Fraser and Zenclussen, 2022). Therefore, they are more aggressive and exhibit a pro-inflammatory phenotype. It can be assumed that the endometrium evolves, differentiates, and undergoes transformation from the moment of ovulation/egg collection to the implantation window. This maturation process manifests as an increase in the number of uterine-type NK cells (CD56 ++) and a decrease in the percentage of HLA-DR and CD16 positive NK cells. Therefore, we termed this type of endometrial immune phenotype with an increased percentage of HLA-DR-positive NK, a high quantity of CD16 positive NK, and a decreased proportion of CD56bright NK, as not transformed.

4.3. Increased CD158a on uNK

The percentage of CD158a positive uNK was significantly higher in women with RIF than in GC. Increased expression of CD158b, CD94 (secretory phase), and CD158a (proliferative phase) by endometrial NK cells from infertile women has been previously observed by McGrath et al. (2009). CD158a, or KIR2DL1, is an inhibitory receptor from the KIRs family that is expressed by NK cells and some subsets of T cells. These receptors bind to HLA-C molecules in trophoblast. Thus, the balance between activatory and inhibitory signals from NK receptors is thought to be critical for preventing maternal rejection of the embryo. Therefore, higher expression levels of CD158a, which exerts an inhibitory influence on NK cells, may affect implantation by suppressing local NK cell activity (e.g., cytokine and growth factor secretion). However, given that we have investigated the expression of only one of many KIRs, and that these receptors are highly polymorphic and are affected by maternal and paternal HLA-C differences, the significance of this finding for the impact on implantation and pregnancy outcomes needs further research.

4.4. Quantity of uNK cells

The mean uNK levels did not differ significantly in our study. The literature is contradictory regarding the number of uNK cells in patients with RIF. A recent meta-analysis by Von Woon et al. (Von Woon et al., 2022) was devoted to uNK levels measured by immunohistochemistry and flow cytometry in patients with recurrent miscarriages and RIF. In most investigations, the levels of CD56 + cells in the endometrium of patients with RIF were higher than those in controls. The significance of this difference disappeared when studies with hormonal medications and male infertility were excluded. In several studies, uNK levels did not differ or were even decreased in RIF patients (Jiang et al., 2017; Donoghue et al., 2019; Babayeva et al., 2020). Ledee et al., (2004, 2016) demonstrated that in some patients, a high quantity of uNK was observed, whereas others had a low quantity of uNK. The two groups had different immune profiles and required different treatment strategies. Such heterogeneous results may be due to different criteria for patient selection and diverse causes of implantation failure. It is also obvious that different methods of evaluation (flow cytometry or immunochemistry), lack of procedure standardization, such as the day of the cycle, type of the cycle (natural, artificial, etc.), and the fact that the endometrium could be taken from different areas of the uterine cavity, thus, may contain different proportions of stroma, glands, and luminal epithelium, could be the cause of the discrepancy in the results.

4.5. T cells, NKT cells

The part of HLA-DR positive endometrial T cells (mainly Th) was increased in RIF patients in our study. High percentage of HLA-DR positive Th cells was seen in all patients with not transformed immune phenotype. In some cases, this sign occurred as a separate feature in women with RIF. HLA-DR is typically expressed by antigen-presenting cells and is associated with antigen presentation. However, some subsets of T cells also express HLA-DR. It has been associated with T cells activation (Holling et al., 2004). HLA-DR expression on effector T lymphocytes upon their activation has been described in autoimmune diseases, viral infections (Saraiva et al., 2021). In a recent study by Tippalagama et al. (Tippalagama et al., 2021), significantly higher expression levels of HLA-DR were associated with antigen-specific T cells activation and inflammation in acute tuberculosis. Authors found that HLA-DR+CD4 + T cells were proliferative, expressed markers associated with an effector memory phenotype, and increased expression of cytotoxic molecules and pro-inflammatory cytokines. Thus, it can be assumed that endometrial Th in RIF patients show excess pro-inflammatory activity not typical for the implantation window.

Similar to Marron and Harrity (2019), we also found that the proportion of NKT-like cells, such as CD3 +CD16 + and CD3 +CD158a+ , was increased in patients with RIF. Most T lymphocytes in peripheral blood are mature αβTCR+CD3 +CD4 + or CD8 + cells. Meanwhile, other forms of T cells, invariant, or type one NKT (iNKT, INKT), type two

NKT (IINKT), NKT-like cells could be found in tissues (Godfrey et al., 2004; Sun et al., 2019). Type INKT cells are known for their potential to respond very quickly to danger, stress signals, and pro-inflammatory cytokines. Upon activation, they immediately transform into effectors releasing large amounts of cytokines and chemokines. Type INKT cells are also responsible for the transactivation of many lymphocytes and leukocyte subpopulations: they promote T cell activation and differentiation, macrophage polarization, NK, dendritic cell and B cell activation, and neutrophil recruitment as well. According to their cytokine expression pattern, subpopulations of iNKT cells are similar to T cell subsets: Th1-like, Th2-like, and Th17-like iNKT cells. Maternal first trimester decidual tissue showed significant enrichment of iNKT cells with a bias toward pro-inflammatory activity (Miko et al., 2021). Activation of type IINKT cells is not fully understood, but rapid-acting after activation is also a major characteristic of type IINKT cells, with the secretion of a wide range of cytokines and transactivation of other immune cells (Miko et al., 2021). Type IINKT cells are found to be functionally distinctive from type INKT cells. They also accumulate in the decidua but exert Th2 type response via cytokine secretion such as IL4, IL10, IL25, and IL17B (Miko et al., 2021). Little is known about the association between iNKT and implantation failure or pregnancy complications. The involvement of decidua iNKT in the pathogenesis of sporadic miscarriages was not found (Shimada et al., 2006). Elevated, decreased or unchanged type IINKT cell ratios were reported to be beneficial for successful implantation and pregnancy after ART (Yuan et al., 2015; Dong et al., 2017; Liu et al., 2019; Miko et al., 2021). It is believed, that NKT and NKT-like cells play important regulatory roles in immunity, including tumor immunity and autoimmunity (Godfrey et al., 2004; Zarobkiewicz et al., 2021).

Given that we did not examine other markers on NKT-like cells in our investigation, we cannot say for sure which category they belong to. Therefore, the significance of these findings is unclear and T cells phenotyping in RIF needs further investigation.

4.6. IL18, IL15, TWEAK, Fn14 and IL18/TWEAK, IL15/Fn14 m-RNA expression

IL18, IL15, TWEAK, Fn14, IL18/TWEAK, and IL15/Fn14 mRNA expression data we obtained do not allow us to distinguish the different types of endometrial immune profiles in patients with RIF, as Ledee et al., (2016, 2020a, 2020b) did. This may be explained by the smaller number of women in the groups, different test systems, and patient selection criteria. However, TWEAK and Fn14 mRNA expression and the ratio of IL18/TWEAK and IL15/Fn14 in RIF patients and GC differed significantly. RIF patients experienced more cases of too low or too high IL18 expression, reduced average levels of TWEAK and Fn14, and increased IL18/TWEAK and IL15/Fn14 ratios.

IL18 is a cytokine whose expression increases during the implantation window. Interaction with angiopoietin-2 causes destabilization of spiral arteries, contributing to their remodeling, necessary for successful implantation. It is also known that at high concentrations, IL18 acts as a pro-inflammatory cytokine, reflecting local production of IFN-γ and tumor necrosis factor alpha, which can cause the activation of uNK and their transformation into cytotoxic cells (Ingram et al., 2009). At low but sufficient concentrations, IL18 contributes to the shift of the cytokine balance to the Th2 type. Meanwhile, TWEAK and its receptor Fn14 act as immune regulators of the Th1/Th2 cytokine balance in the human endometrium (Winkles, 2008). High expression of TWEAK in the endometrium neutralizes the action of high levels of IL18 and prevents the transformation of mature uNK into dangerous cytotoxic cells (Ingram et al., 2009). We observed that low TWEAK and IL18 expression were associated with not transformed immune phenotype of the endometrium. Therefore, low TWEAK expression in combination with a low level of IL18 most likely deepens the deregulation of the immune picture of the endometrium, hindering its transformation and preserving the pro-inflammatory type of environment, resulting in the impossibility for uNK to losing HLA-DR and CD16 and becoming CD56 bright cells. In our study, we did not observe any correlation between flow cytometry data and IL15 mRNA. However, we observed a decrease in Fn14 mRNA expression and an increase in the IL15/Fn14 mRNA ratio. Indeed, IL15 plays an important role in implantation. It contributes to the recruitment and expansion of uNK cells (Carson et al., 1994; Ledee et al., 2016; Gordon, 2021; Xie et al., 2022). In three cases with low amounts of uNK (less than 25 %), IL15 mRNA expression was also relatively low, although similar levels occurred in women of both groups (RIF and GC) with normal levels of CD56 + cells. Fn14 is the membrane receptor of TWEAK and is expressed by almost all cells (with the exception of primary T and B lymphocytes). This indicates that Fn14 function and its interaction with TWEAK are basic, universal processes that occur in different tissues. Thus, it is known that Fn14 is an early response gene; its expression increases after tissue damage and plays an important role in a coordinated complex process involving cell migration, inflammation, and angiogenesis (Winkles, 2008). Prolonged overload of the TWEAK-Fn14 system or excessive expression of Fn14 may be involved in the development of acute ischemic stroke, rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, and cancer. The relative decrease in Fn14 expression observed in patients with RIF may also be a link in the endometrial cell dysfunction chain.

Thus, RIF patients have certain differences in the endometrial immune cell phenotype, which can be detected using various research methods (flow cytometry and PCR gene expression). In 30 % of patients, these findings correlate with each other and form a picture of a not transformed immune phenotype of the endometrium hostile to the embryo.

5. Conclusions

- Immune abnormalities of the endometrium (atypical for fertile control) were detected in more than half (66.7 %) of the patients with recurrent implantation failures after the genetically tested embryo transfers.
- In one-third of the cases (30 %), there was a unique immune profile of the endometrium, which we called the not transformed endometrial immune phenotype. It was characterized by a combination of features such as high expression of HLA-DR on NK cells, increased fraction of CD16 + cells among NK cells, and decreased fraction of CD56bright cells.
- In general, patients with recurrent implantation failures were characterized by an increased HLA-DR, CD16, CD158a expression on uterine NK, decreased number of T cells, increased number of Th and HLA-DR expressing Th, decreased Tc fraction, increased CD158a and CD16 expression on T lymphocytes (NKT-like cells).
- Compared with gestational carriers, patients with recurrent implantation failures had a greater discrepancy in IL18 mRNA expression, decreased average levels of TWEAK and Fn14, and increased IL18/TWEAK and IL15/Fn14 ratios. Decreased TWEAK and IL18 mRNA expression is associated with a not transformed endometrial immune phenotype.

CRediT authorship contribution statement

Iryna Sudoma – data collection and analysis, manuscript writing. Yana Goncharova – data collection and analysis, manuscript-writing assistance Boris Dons'koy – immunological study, data collection and analysis. Dmytro Mykytenko – genetical study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jri.2023.103943.

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