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Article

Different behavior of NK cells isolated from healthy women and women with recurrent spontaneous abortion after treatment with human amniotic epithelial cells

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Abstract

Maternal immunotolerance during pregnancy is heavily dependent on the critical properties of human amniotic epithelial cells (hAECs). Recurrent spontaneous abortion (RSA) is one of the most common diseases in women and is caused by feto-maternal immunotolerance disruption. The objective of this study is to investigate how hAEECs affect pNK cells isolated from RSA and healthy women in terms of immunomodulation. Peripheral blood NK cells were isolated from 20 women with RSA and 20 healthy women. Purified NK cells were co-cultured with hAECs, obtained from full-term healthy pregnant women at different cellular ratios. After 24 and 72 h of incubation, the expression of immunomodulatory genes in hAECs, immunophenotype, and cytotoxicity of NK cells, and cytokine production were investigated using real-time PCR, flow cytometry, and ELISA techniques, respectively. We observed a significant increase in TGF-β and IL-10 production, and $CD56^{bright}$ $CD16^{+}$ subpopulation in pNK cells, a significant decrease in IFN-γ production and CD107a and FasL expression on NK cells. Also, NK cells' cytotoxicity against K562 cells was diminished after co-culture with hAECs. The expression of *TGF-β* and *HLA-G* genes by hAECs was diminished after co-culture with NK cells isolated from women with RSA. Our research indicates that the interaction between NK cells and hAECs influences the phenotype and function of both cells. Also, NK cells belonging to women with RSA and healthy women exhibit different behavior during treatment with hAECs, possibly due to NK cell dysfunction. However, extensive research is required to assess NK cell defects and their mutual interaction with hAECs.

Keywords: human amniotic epithelial cell, immunomodulation, natural killer cell, recurrent spontaneous abortion

1. Introduction

Human amnion membrane has been studied for a long time and has various clinical applications.[1,2] The placenta's innermost layer near the semiallogeneous fetus contains this membrane, which has anti-inflammatory and immunomodulatory properties.[3–5] These properties are critical for maternal immunotolerance during pregnancy. These properties play a crucial role in maternal immunotolerance during pregnancy.[6] The amnion membrane is home to one of the most extensively studied stem cells, known as human amniotic epithelial cells (hAECs). Many unique characteristics of the amnion membrane are attributed to these cells,[7] and during the last 2 decades, they have been investigated for their therapeutic potential in different pathological conditions.[8–16] Also, these cells can be released into amniotic fluid while showing epithelial features and expressing epithelial surface antigens.[17,18] Considering the crucial role of hAECs during pregnancy, recurrent abortion is a noteworthy area for exploring the therapeutic potential of these cells.

Recurrent spontaneous abortion (RSA) refers to a pregnancy failure that occurs 3 or more times before 20 wk of gestation.[19] One to three percent of women of reproductive age are affected by RSA, which is one of the most common diseases in women.[20] RSA pathogenesis is a complex process that can be caused by multiple factors, such as hormonal imbalances, genetic predisposition, infections, and environmental issues.[21] Dysregulation of feto-maternal immunotolerance is the cause of abortion in around 50% of cases.[22] The precise crosstalk of maternal immune cells, including innate lymphoid cells (ILCs), T cells, and trophoblasts, takes place during normal pregnancy.[23] Natural killer (NK) cells are pivotal ILCs crucial for implantation and early placental growth.[24] NK cells operate Ca_2^+-dependent granule exocytosis, exert granule-independent cytotoxicity via Fas/Fas ligand (FasL) interaction, and release cytotoxic proteins and membrane-bound or secreted cytokines during activation to induce target cell death.[25] Decidual natural killer (dNK) cells have a cytotoxic capacity, and any change in the regulation of their cytotoxic activity turns them into harmful cells, which can lead to RSA.[26,27]

Although 90% of uterine NK (uNK) cells are $CD56^{bright}CD16^{dim/+}$ with low cytotoxic capacity, they produce higher levels of

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cytokines.[28] The decrease in uNK cells in subjects with RSA compared to fertile controls suggests that RSA is closely associated with an abnormal proportion of uNK cells.[29] These cells control the proliferation and invasion of trophoblasts.[30,31] HLA class Ib, HLA-G, and HLA class Ia molecules are low-level expressed in hAECs, whereas HLA class II molecules are not expressed.[32,33] Therefore, hAECs exhibit immunomodulatory effects on the feto-maternal interface. With these premises, our study aimed to assess the immunomodulatory effects of hAECs on NK cells isolated from RSA and healthy women.

2. Materials and methods

2.1 Ethics statement

The study was approved by the Ethics Committee of the Isfahan University of Medical Sciences (code: IR.MUI.MED.RC.1398.662), and all participants provided informed written consent before enrollment in this study.

2.2 Study population and sampling

The pilot study compared hAECs isolated from normal pregnant women (n = 5) and pregnant women with a history of RSA (n = 5). The levels of IL-10 and TGF produced by hAECs were compared between the RSA and normal groups (Supplementary Table S1). No significant differences were found between the 2 groups (Supplementary Fig. S1).

Consequently, hAECs from normal pregnant women were selected for co-culturing with NK cells for the main study. Normal and RSA women's NK cells were co-cultured with normal hAECs, and the cell interactions were subsequently evaluated (Supplementary Fig. S1).

For isolation of hAECs, 20 full-term placenta units were collected after elective cesarean sections at Shahid Beheshti Hospital, Isfahan, Iran. The samples were collected from mothers (n = 20) who were healthy and had a normal singleton pregnancy without any history of abortion or infertility treatments. They completed a written consent form prior to enrolling in the current study.

A total of 20 women from RSA were examined by an immunology laboratory and immunotherapy center in Isfahan, Iran as part of the study population. Any anatomical or physiological abnormalities and infectious diseases leading to infertility and pregnancy loss were excluded according to World Health Organization criteria.[34] Blood sampling from RSA women was performed before immunotherapy and corticosteroid therapy. Women in RSA underwent blood sampling before immunotherapy and corticosteroid therapy. The control group consisted of 20 healthy women who were age- and BMI-matched, had at least 1 live birth, and had no history of abortion. To isolate NK cells, 50 ml of blood was collected from all participants.

2.3 Isolation, characterization, and culture of hAECs

The placenta was transferred to the cell culture room in a sterile receptacle filled with ice-cold phosphate-buffered saline (PBS; Biosera, France). All cell isolation steps were carried out in sterile conditions. The amnion membrane was removed from both the placenta and the chorion manually. To eliminate blood clumps and cellular debris, the membrane was cut into smaller pieces and flushed with PBS multiple times. Trypsin-EDTA (0.05%) (Gibco, USA) was used to digest the membrane pieces for 20 min at 37 °C with mild shaking (50 RPM) for 20 min. The second and third enzymatic digestions were performed for 30 min at 37 °C with gentle shaking. Isolated cells were centrifuged at 200 × *g* for 5 min at 4 °C. The cell pellets were re-suspended in 10 ml DMEM/F12 culture medium (Biosera, France) and filtered through a 100 μm cell strainer (Thermo Fisher Scientific, USA). The phenotypic characteristics and purity of freshly separated cells were evaluated using flow cytometry. Cells with 95% purity were considered hAECs. Intracellular staining with Alexa Fluor 488 anticytokeratin (Biolegend, USA) was performed according to the manufacturer's instructions (eBioscience, USA). The cells were stained with different antibodies (BioLegend, USA), including fluorescein isothiocyanate (FITC) conjugated antihuman CD2, FITC-CD3, FITC-CD8, FITC-CD33, FITC-CD57, FITC-CD14, phycoerythrin (PE)-CD66b, FITC-CD45, peridinin–chlorophyll protein complex (PerCP)-CD56; and matched to isotype control IgG for 25 min at 4 °C. The data were acquired operating a FACSCalibur system (Becton–Dickinson, USA) and analyzed utilizing Flowjo software 10.

To assess the allogeneic effects of hAECs, a cellular enzyme-linked immunosorbent assay (ELISA) BrdU (bromodeoxyuridine or 5-bromo-2′-deoxyuridine) kit (Abcam, Germany) was utilized. BrdU labeling was performed in vitro for allogeneic PBMCs and hAECs. In the BrdU assay, BrdU was incorporated into the replicating DNA of proliferating PBMCs and was detected using anti-BrdU antibodies. The proliferative response of PBMCs was measured following the manufacturer's instructions. PBMCs were separated by Lymphoperep (STEMCELL Technologies, USA) density gradient centrifugation. Subsequently, PBMCs that were newly isolated were mixed with hAECs in various proportions (1:1, 1:2, and 2:1) on a 96-well cell culture plate at 37 °C with 5% CO_2. PBMCs that were stimulated with Phytohemaggutinin (PHA, Sigma) were used as positive controls with a final concentration of 5 μg/ml in the well. The negative controls consisted of only unstimulated PBMCs and hAECs. The cultured cells were labeled with anti-BrdU conjugated antibodies. Lastly, the proliferation rate of PBMCs was assessed to determine the allogeneic effects of hAECs. Freshly isolated hAECs were cultured in DMEM/F12 medium (Biosera, France) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 1% penicillin/streptomycin (pen/strep; Sigma, Germany), and human recombinant epithelial growth factor (EGF; PeproTech, USA) at 37 °C, 80% humidity, and an atmosphere of 5% CO_2.

2.4 Magnetic separation of NK cells

The human NK Cells Isolation kit (MACS: magnetic-activated cell sorting) was used to negatively select NK cells from freshly isolated PBMCs under the manufacturer's instructions (Miltenyi Biotec, Germany). Accordingly, non-NK cells, including T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes, and erythrocytes, were magnetically labeled (using a cocktail of biotin-conjugated antibodies and the NK Cell MicroBead Cocktail). Subsequently, the cells that had been separated were labeled with the FITC-antihuman CD3 monoclonal antibody (BioLegend) and the PE-antihuman CD16/56 antibody cocktail (BD Biosciences). The appropriate isotype control antibodies (BioLegend, USA) were used as a negative control. Cell purity was analyzed with a FACSCalibur flow cytometer system (BD Biosciences, San Jose, CA). $CD3^-$ $CD16/56^+$ cells were categorized as NK cells, and only samples with a purity of >90% were permitted for culture.

The pure NK cells were cultured in Stem Cell Growth Medium (SCGM; CellGenix, USA) enriched with 10% fetal bovine serum (FBS, Gibco, USA), 1% penicillin/streptomycin (pen/strep, Sigma, Germany), and 100 units/ml interleukin 2 (IL-2, eBiosciences, USA). The cells were infused in T25 cell culture flasks and kept at 37 °C, 80% humidity, and 5% CO_2 for 2 d.

2.5 Co-culture designing

After 48 h of incubation of hAECs in T75 cell culture flasks, adhered cells were dissociated by trypsin/EDTA 0.25% (Gibco, USA) and seeded at a density of 5×10^5 cells/well in 24-well plates for subsequent experiments. The adhesion of the hAECs was achieved after 12 h of incubation. The preparation of hAEC: NK cell ratios of 2:1, 1:1, and 1:2 was made by seeding NK cells in different numbers in the test wells. In the co-culture system, negative controls were achieved by plating 5×10^5 hAECs and NK cells alone in certain wells. The cultured cells spent 24 or 72 h incubating at 37 °C with 5% CO_2. After each incubation interval, supernatants (for cytokines assay), NK cells (for flow cytometry analysis), and hAECs (for gene expression analysis) were harvested separately.

2.6 Flow cytometry

The analysis of NK cells' cytotoxicity markers and immunophenotypes was done through flow cytometry. The NK cells (1 to 2×10^5 cells/ml) were stained with monoclonal antibodies, including FITC-conjugated anti-CD69, FITC-conjugated anti-CD107a, FITC-conjugated anti-CD178, FITC-conjugated anti-CD3, PE-conjugated anti-CD16, and PerCp-conjugated anti-CD56 antibodies (BioLegend, USA). Antibodies were incubated with the cells for 25 min at 4 °C and stained as per the manufacturer's protocol. Negative controls were achieved by using the appropriate isotype control antibodies (IgG, BioLegend, USA). NK cells were divided into subsets based on $CD3^-CD16^{-/+}CD56^{bright}$ and $CD3^-CD16^+CD56^{dim}$.

2.7 Cytokines assay

In order to investigate the effect of the interaction of NK cells and hAECs on the production of anti-inflammatory cytokines, the concentration of transforming growth factor beta (TGF-β) and interleukin 10 (IL-10) were investigated in different co-cultured groups. Also, changes in interferon-gamma (IFN-γ) concentration were determined to study the effect of hAECs on NK cells. The concentration of these cytokines was quantified using an ELISA kit according to the manufacturer's protocol (LEGEND MAX™ Human ELISA Kit, BioLegend, USA) in the study groups. ELISA kits detected 2 pg/ml for IFN-γ and IL-10 and 3.5 pg/ml for TGF-β.

2.8 Real-time PCR

The total RNA of hAECs in all co-culture and hAECs negative culture wells was extracted using an RNX-Plus Solution (Sinaclon, Iran), and the purity of the RNA was assessed by spectrophotometry. Reverse transcription of RNA was performed using the Revertaid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Lithuania). All procedures were conducted according to the manufacturer's protocol. The quantitative real-time transcription-polymerase chain reaction (qRT-PCR) was carried out in a 20 µl-reaction mixture containing RealQ Plus 2 × Master Mix Green (Ampliqon, Denmark), cDNA, 0.2 µM of each primer, and nuclease-free water.

The real-time PCR conditions contained an initial denaturation at 95 °C for 15 min, followed by a 45-cycle amplification including denaturation at 95 °C for 30 s, and extension at 61 °C for 60 s pursued by melting curves. The reactions were executed with the assistance of a StepOne Real-time PCR system from Applied Biosystems, USA. Table 1 shows the primer sequences.

2.9 Statistical analysis

Basic characteristics of study participants were reported as mean ± standard deviation (SD) and were compared between 2 groups using independent samples *t*-test. The main continuous study's outcomes were reported as mean ± standard error. The normality of continuous data were evaluated through the Shapiro–Wilk test. Mixed effect and nested analysis of variance (ANOVA) were used to compare the mean values of study outcomes between groups. In our study, the top-level variable was the main groups (women with RSA and healthy women) is a fixed factor, and cellular ratios (1:2, 1:1, and 2:1) and time points (24 and 72 h) were considered as nested random factors; accordingly, data analysis was done under the framework of mixed effects nested ANOVA. The baseline expression levels of each marker in negative control wells were statistically adjusted for each time point and cellular ratio (Figs. 7–9). These adjustments were also performed for other assessed parameters (gene expression and cytokine concentration). Pairwise comparisons were done by using the Bonferroni post hoc test. All statistical analyses were performed utilizing SPSS version 16 (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp) and GraphPad Prism Version 9.0.0.

3. Results

There were no significant differences in age and BMI between the study groups, including women with RSA and healthy women (Table 2). Peripheral blood NK cells were isolated from healthy and RSA women by the MACS isolation method. $CD3^-CD16^+CD56^+$ cells were considered NK cells, and their purity was, on average, over 90%.

The hAECs isolated from the amniotic membrane were investigated concerning phenotype, purity, and lack of immunogenicity. After initial culture, hAECs were characterized by flow cytometry, and as shown in Table 3, they were negative for cell-specific lineage markers and positive for epithelial markers (cytokeratin) (Supplementary Fig. S2). hAECs purity was more than 85% on average. According to the BrdU cell proliferation assay, hAECs did not have any immunogenicity to stimulate a significant proliferative response in allogeneic PBMCs after co-culture at 1:2, 1:1, and 2:1 cellular ratios (PBMC:hAEC). As indicated in Fig. 1, the optical density (OD) of the test and negative control wells was significantly lower compared to the OD of the positive control wells ($P < 0.0001$).

3.1 hAECs increased the $CD56^{bright}$ NK cells in women with RSA after 72 h of co-culture

The percentage of the $CD56^{bright}CD16^{dim}$ subset of NK cells was significantly higher in the healthy group compared to the RSA

Table 1. Primer sequences of GAPDH, HLAG, IL-10, and TGFB1.

Gene	Primer sequences 5′→3′	
	Forward	Reverse
GAPDH	AACAGCCTCAAGATCATCAGC	GTAGAGGCAGGGATGATGTTC
HLAG	CTACTCTCAGGCTGCAATGTG	TTGAGACAGAGACGGAGACATC
IL-10	GCGCTGTCATCGATTTCTTC	TATAGAGTCGCCACCCTGATG
TGFB1	AAGTGGACATCAACGGGTTC	CAATGTACAGCTGCCGCAC

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HLAG, human leukocyte antigen G; IL-10, interleukin 10; TGFB1, transforming growth factor beta 1.

Table 2. Demographic characteristics of participants in the study groups.

Criteria	RSA group Healthy group		P value
	Mean ± SD (min–max)		
Age (year)	28.9 ± 3.75 (23 to 35)	27.7 ± 3.7 (23 to 35)	0.32 (ns)
BMI (kg/cm^2)	26 ± 0.7 (24 to 27)	25.8 ± 1.1 (24 to 27)	0.49 (ns)
Number of live birth	1.05 ± 0.9 (0 to 3)	2.05 ± 0.7 (1 to 3)	—
Number of miscarriage	3.7 ± 1.3	0.0	—

RSA, recurrent spontaneous abortion; BMI, body mass index; ns: $P > 0.05$.

Table 3. Phenotypic characterization and the expression level of lineage markers in hAECs.

Markers of cell lineage	Sample 1	Sample 2	Sample 3
T cell			
CD2	Negative	Negative	Negative
CD3	Negative	Negative	Negative
CD4	Negative	Negative	Negative
CD8	Negative	Negative	Negative
Monocyte			
CD14	Negative	Negative	Negative
B cell			
CD38	Negative	Negative	Negative
Granulocyte			
CD33	Low/Negative	Low/Negative	Low/Negative
CD45	Negative	Negative	Negative
CD66b	Negative	Negative	Negative
NK cell			
CD16	Negative	Negative	Negative
CD56	Negative	Negative	Negative
CD57	Negative	Low/Negative	Low/Negative
Epithelial cell			
Cytokeratin	High	High	High

Negative: 0% to 1% expression, Low/Negative: <30% expression, and High: >30% expression.

group (Fig. 2), and the population of these cells increased significantly after 72 h of co-culture with hAECs at different ratios (14.91% ± 0.86% vs 34.96% ± 2.63%, $P < 0.0001$ and 11.71% ± 0.26% vs 30.83% ± 1.57%, $P = 0.0078$, respectively for 1:1 and 2:1 ratios) (Fig. 2B).

The CD56^{dim}CD16^{bright} subset of NK cells considered the predominant phenotype in women with RSA, was significantly higher in this group compared to the healthy group after 24 h of incubation in all cellular ratios (2.6% ± 0.13% vs 11.43% ± 0.51%, 2.45% ± 0.09% vs 15.07% ± 0.53%, 2.23% ± 0.11% vs 14.98% ± 0.57%, $P < 0.0001$, respectively) (Fig. 3A); 72-h co-culture resulted in a significant decrease in the CD56^{dim}CD16^{bright} cell subset in the RSA group (11.43% ± 0.51% vs 2.47% ± 0.37%, 15.07% ± 0.53% vs 2.13% ± 0.15%, 14.98% ± 0.57% vs 2.03% ± 0.32%, $P < 0.0001$, respectively), but we observed no similar decline in the healthy group (Fig. 3B).

3.2 hAECs decreased the CD107a expression on the surface of NK cells

In the present study, we evaluated modifications of the expression of NK cytotoxicity markers, including CD69, FasL (CD178), and CD107a, after co-culture with hAECs. The expression of these cytotoxicity markers on NK cells isolated from the RSA group was significantly higher compared to the healthy group.

The expression of CD69 and FasL was significantly increased affected by hAECs compared to negative controls (single culture of

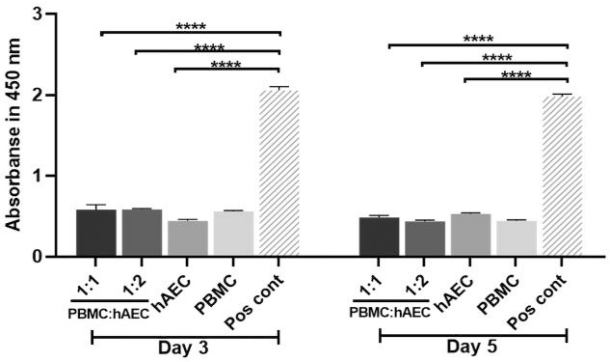

Fig. 1. Lack of proliferative response in PBMCs due to immunogenicity of hAECs; hAECs co-cultured with PBMCs at various ratios for 3 and 5 d did not induce a significant proliferative response. hAEC, human amniotic epithelial cell; PBMC, peripheral mononuclear cell; Pos cont, positive control. **** $P < 0.0001$.

NK cells) (Figs. 4A and 5A). The expression of CD69 in the RSA group showed an increasing trend over time (36.1% ± 0.22% vs 16.25% ± 0.45%, $P < 0.0001$ and 34.08% ± 0.26% vs 18.01% ± 0.53%, $P < 0.0001$, respectively for 2:1 and 1:1 ratios) (Fig. 4B), and after 72 h of incubation, its expression in the RSA group was significantly higher compared to the healthy group (35.99% ± 0.34% vs 18.9% ± 0.35%, $P = 0.0018$ and 33.94% ± 0.3% vs 18.29% ± 0.34%, $P = 0.0051$, respectively for 2:1 and 1:1 ratios) (Fig. 4C).

About the expression of FasL, we observed a significant increase in both study groups after co-culture compared to negative control wells (Fig. 5A). Of course, the expression of FasL in the healthy group significantly decreased over time (4.7% ± 0.05% vs 4.08% ± 0.005%, $P = 0.015$ and 6.21% ± 0.21% vs 4.79% ± 0.028%, $P = 0.0026$, respectively for 2:1 and 1:1 ratios) (Fig. 5C).

The expression of CD107a decreased significantly affected by hAECs in the RSA group, while we did not find significant CD107a expression changes in the healthy group (Fig. 6A). CD107a expression in the RSA group significantly decreased after 72-h co-culture (20.22% ± 0.24% vs 15.58% ± 0.15%, $P = 0.026$ and 18.87% ± 0.22% vs 14.47% ± 0.14%, $P = 0.03$, respectively for 2:1 and 1:1 ratios), while no significant change was observed in the healthy group (Fig. 6B). According to Fig. 6, our findings indicate that NK cells isolated from women with RSA exhibit greater susceptibility to hAECs. This trend was consistent across all cellular ratios and both time intervals.

3.3 hAECs decreased the cytotoxicity of NK cells against target cells

After 72 h of culturing in the presence and absence of hAECs, NK cells were incubated with K562 cells (target cell, Fig. 7A) at cellular ratios of 1:20 (E:T) and 1:10 to assess the NK cell-mediated cytotoxicity. As shown in Fig. 7B, the percentage of K562 cells that underwent early and late apoptosis was determined by flow cytometry using the Annexin V/PI labeling method. NK cells affected by hAECs exhibited lower cytotoxicity compared to negative control wells. Significant differences were observed in both study groups and all cellular ratios, but no significant difference was recorded between the RSA and healthy groups (Fig. 7C).

3.4 NK cells decreased *HLA-G* and *TGF-β* gene expression in hAECs

In normal conditions, hAECs have a stable gene expression of *TGF-β*, *HLA-G*, and *IL-10*. The expression of these genes is related

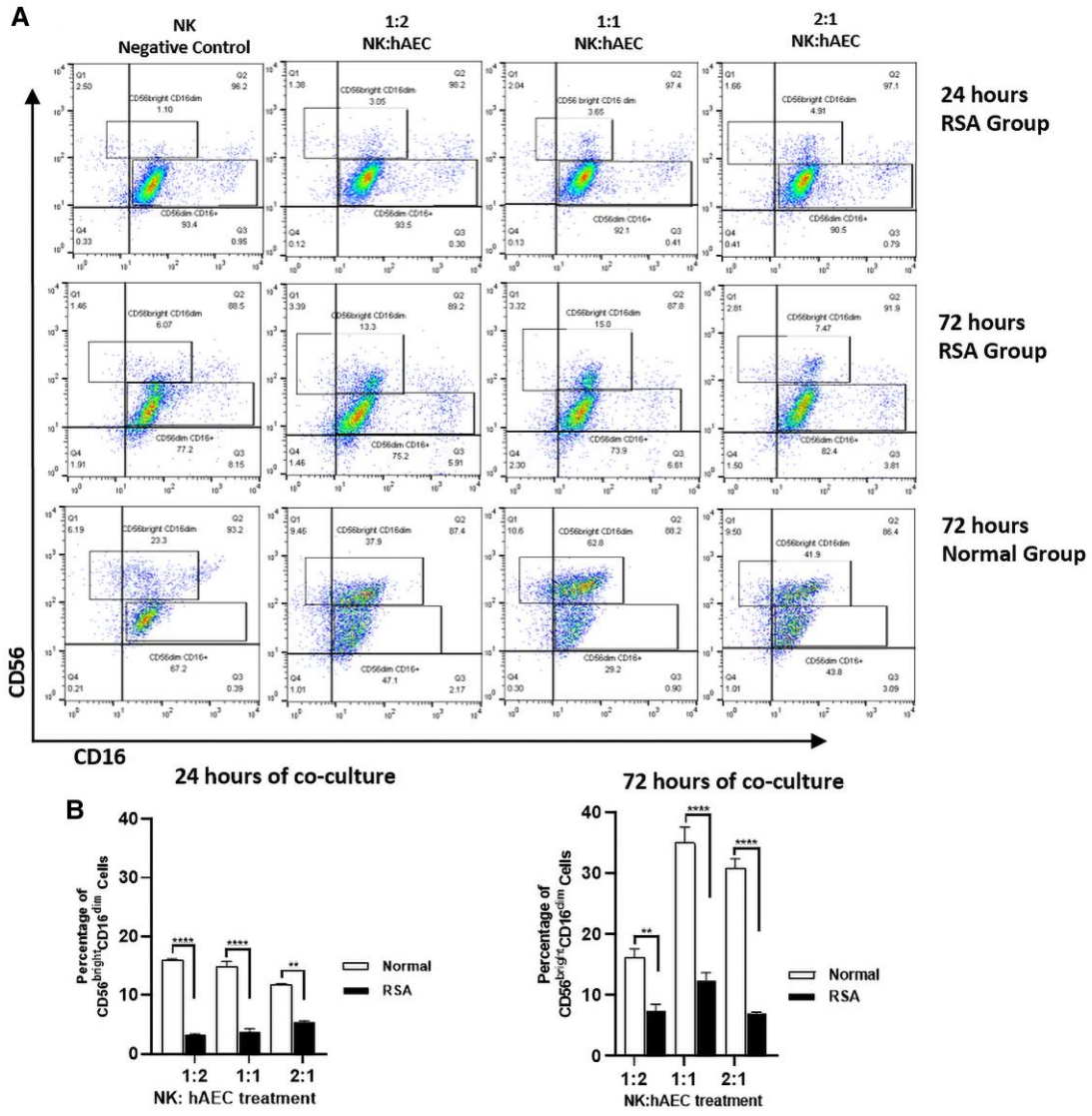

Fig. 2. Changes in the population of $CD56^{bright}CD16^{dim}$ and cells after co-culture with hAECs; A) Flow cytometry plots related to the strategy of gating for determining $CD56^{dim}CD16^{+}$ and $CD56^{bright}CD16^{dim}$ subsets of NK cells. B) The percentage of $CD56^{bright}CD16^{dim}$ cells was significantly higher in the healthy group compared to the RSA group, and this increase was observed in both 24 and 72-h co-cultures (n = 20). Significant differences existed between the 1:2, 1:1, and 2:1 cellular ratios. The percentage of $CD56^{bright}$ $CD16^{dim}$ NK cells increased over time in both groups. NK, natural killer cell; hAEC, human amniotic epithelial cell; RSA, recurrent spontaneous abortion. **P < 0.01, and ****P < 0.0001.

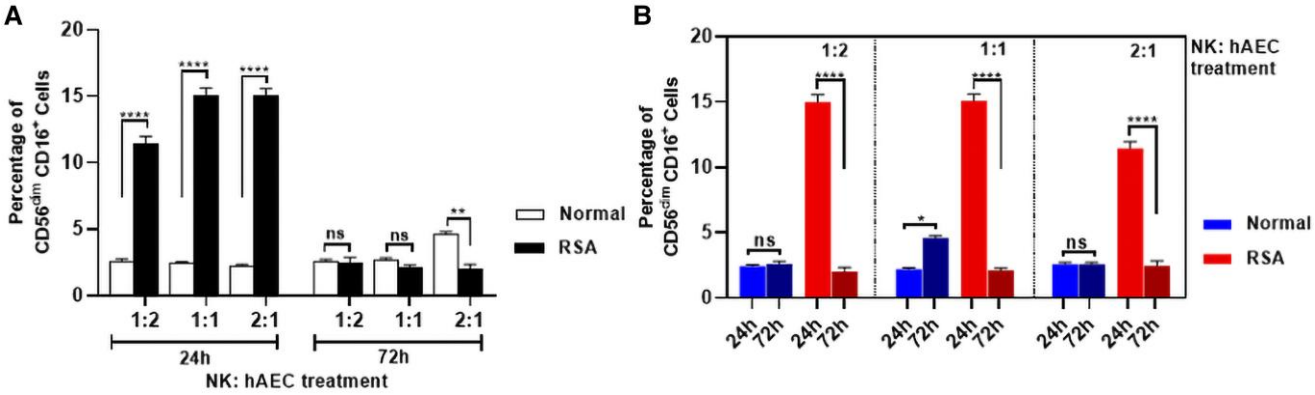

Fig. 3. Differences in the population of $CD56^{dim}CD16^{+}$ NK cells in the study groups; A) In the 24-h co-cultures, the percentage of $CD56^{dim}$ $CD16^{+}$ cell population was significantly higher in the RSA group compared to the healthy group, but the difference between the 2 study groups decreased after 72 h (n = 20). B) The population of $CD56^{dim}$ $CD16^{+}$ cells in the RSA group significantly decreased after 72 h of co-culture with hAECs compared to 24-h co-cultures (n = 20). Changes in the population of these cells were not statistically significant in the healthy group. NK, natural killer cell; hAEC, human amniotic epithelial cell; RSA, recurrent spontaneous abortion. ns: P > 0.05, *P < 0.05, **P < 0.01, and ****P < 0.0001.

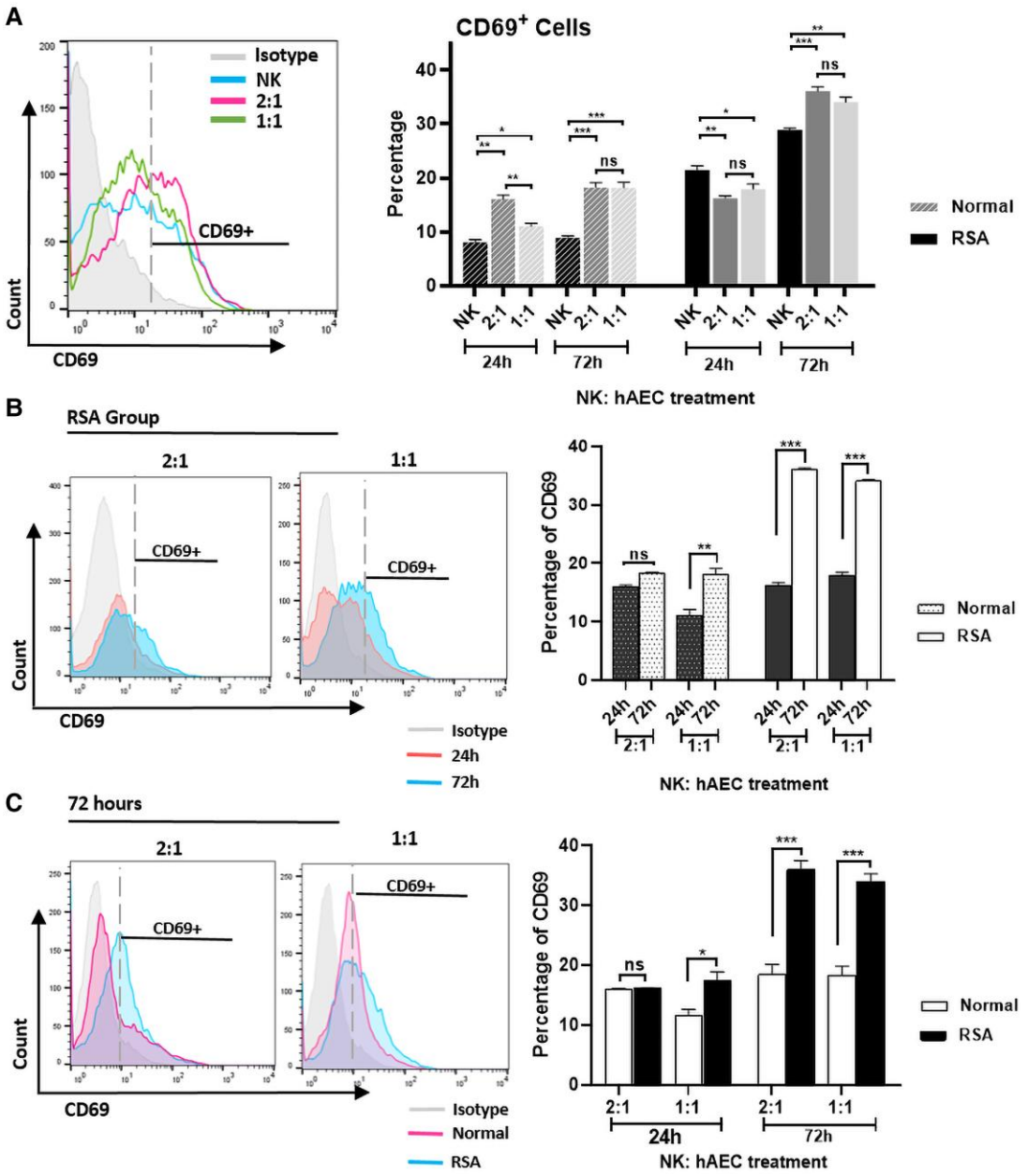

Fig. 4. CD69 expression on NK cells after co-culture with hAECs differed in the study groups. A) The histogram displays the expression level of CD69 on the cell surface. The expression of CD69 on the surface of NK cells in the healthy group at 24 and 72 h and in the RSA group at 72 h after co-culture with hAECs significantly increased compared to the single culture of NK cells (n = 20). B) The expression of the CD69 on NK cells in the RSA group (n = 20) was higher compared to the healthy group. C) The expression of CD69 on NK cells after 72 h of co-culture increased compared to 24-h co-culture. NK, natural killer cell; hAEC, human amniotic epithelial cell; RSA, recurrent spontaneous abortion. ns: $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

to the immunomodulatory function of hAECs, and some pro-inflammatory factors can result in suppressing their expression. In the present study, we evaluated the expression changes of these genes in hAECs after co-culturing with NK cells isolated from women with RSA and healthy women at different cellular ratios. *GAPDH*, which has a high and stable expression level in hAECs, was considered a housekeeping gene.

The *HLA-G* gene expression differences in different cellular ratios in study groups are shown in Fig. 8A. As presented in Fig. 8A, co-culture of hAECs and NK cells significantly decreased the expression of the *HLA-G* gene in study groups compared to negative control wells (single culture of hAECs). NK cells isolated from women with RSA caused a more significant decline (1.72-fold and 12.8-fold reduction) in the expression of the *HLA-G* gene within the initial incubation interval at cellular ratios of 1:1 (0.31 ± 0.01 fold vs 0.18 ± 0.02 fold, $P = 0.02$) and 2:1 (0.32 ± 0.01 vs 0.025 ± 0.002 fold, $P < 0.0001$) (Fig. 8B). After 72 h of co-culture, a significant decrease (3.45-fold) in the RSA was observed at the 2:1 cellular ratio (0.21 ± 0.01 fold vs 0.06 ± 0.003 fold, $P = 0.04$) in compared healthy groups (Fig. 8B).

NK cells isolated from women with RSA caused a decrease in the *TGF-β* gene expression in hAECs at both incubation intervals (Fig. 8C). The *TGF-β* gene expression in the RSA group at 72 h of co-culture (at 1:1 cellular ratio) significantly increased (2.08-fold) compared to 24-h co-cultures (0.12 ± 0.01 fold vs 0.25 ± 0.01 fold, $P < 0.0001$). In the healthy group, the 24-h co-culture of hAECs with NK cells at the 2:1 cellular ratio caused a significant increase (1.73-fold) in the expression of the *TGF-β* gene compared to the 1:1

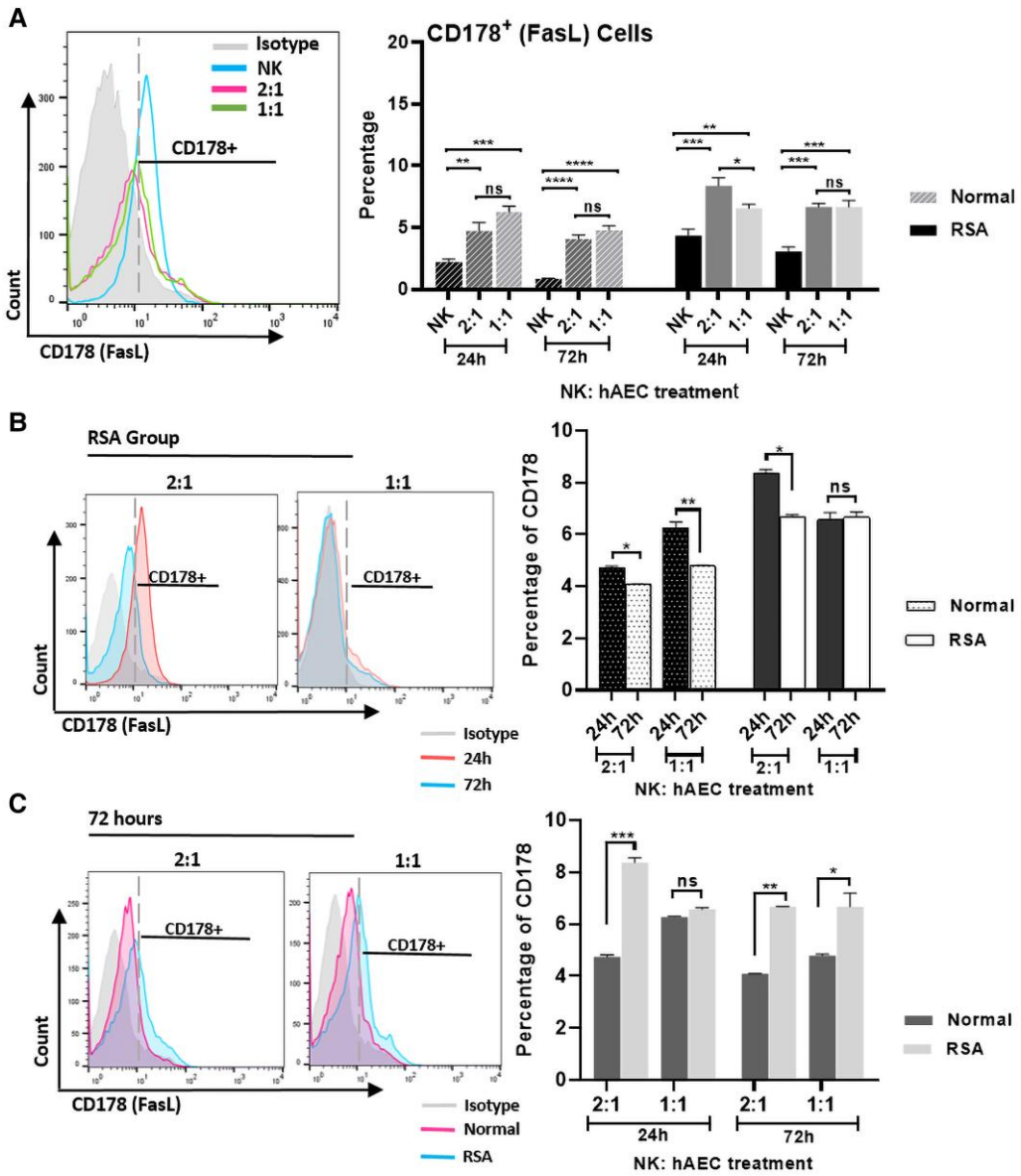

Fig. 5. Comparison of FasL expression on NK cells after co-culture with hAECs between the study groups; A) the histogram reveals the expression level of FasL on the NK cells' surface. As a result of the co-culture in the healthy and RSA groups, the expression of FasL significantly increased (n = 20). B) FasL expression on NK cells in the RSA group (n = 20) was higher compared to the healthy group. C) After 72 h of co-culture, FasL expression significantly decreased. The decrease in the RSA group was not significant for the 1:1 cellular ratio. NK, natural killer cell; hAEC, human amniotic epithelial cell; RSA, recurrent spontaneous abortion. ns: $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

cellular ratio (1.26 ± 0.09 fold vs 0.73 ± 0.03 fold, $P = 0.0034$). However, with incubation up to 72 h, the expression of this gene significantly decreased (1.71-fold) in the 2:1 cellular ratio compared to the 1:1 cellular ratio (0.21 ± 0.01 fold vs 0.36 ± 0.009 fold, $P = 0.0004$). In the healthy group, the gene expression of the *TGF-β* significantly diminished in both cellular ratios over time. Therefore, based on our results presented in Fig. 8C, the trend of changes in the expression of this gene was different in the study groups. Initially, the gene expression of *TGF-β* in the healthy group was significantly higher compared to the RSA group (0.73 ± 0.037 fold vs 0.006 ± 0.0005 fold, $P < 0.0001$ and 1.26 ± 0.09 vs 0.87 ± 0.004 fold, $P < 0.0001$) (Fig. 8D), and up to 72 h of co-culture, just at 2:1 cellular ratio, *TGF-β* expression in the healthy group increased significantly (3.33-fold) compared to RSA group (0.2 ± 0.01 fold vs 0.06 ± 0.003 fold, $P = 0.0019$) (Fig. 8D).

The gene expression of the *IL-10* in hAECs did not show a consistent pattern of increase or decrease at different cellular ratios and time intervals. The significant increases in gene expression of *IL-10* were 1.09 ± 0.1 fold vs 1.8 ± 0.25 fold, $P = 0.019$ and 0.39 ± 0.04 fold vs 1.02 ± 0.13 fold, $P = 0.024$, respectively for 1:1 and 2:1 ratios in the RSA group and 0.16 ± 0.015 fold vs 1.92 ± 0.15 fold, $P = 0.0002$ and 0.48 ± 0.049 fold vs 1.49 ± 0.13 fold, $P = 0.0008$, respectively for 1:1 and 2:1 ratios in the healthy group (Fig. 8E). However, after 72 h of co-culture, the expression of this gene at 2:1 cellular ratio in the RSA group significantly decreased (1.02 ± 0.13 fold vs 1.49 ± 0.13 fold, $P = 0.0017$) (Fig. 8F).

3.5 The co-culture of hAECs and NK cells stimulated the production of TGF-β and IL-10 in both cell types and decreased the production of IFN-γ in NK cells

The concentration of TGF-β, IL-10, and IFN-γ cytokines were measured in the supernatants of co-culture and negative control

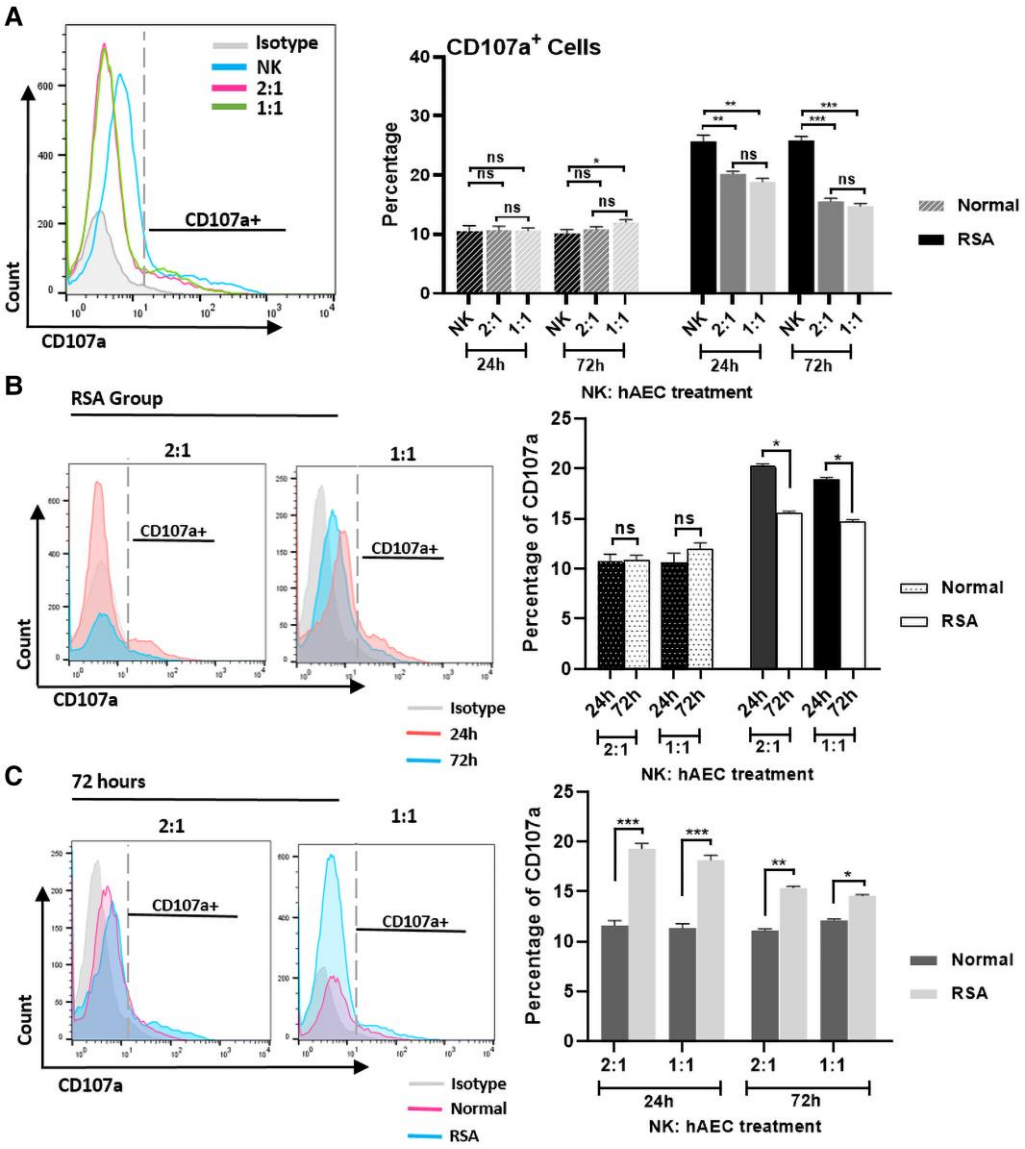

Fig. 6. CD107a expression on the surface of NK cells in the RSA group was significantly higher compared to the healthy group. A) The histogram shows the expression level of CD107a. The expression of CD107a on the surface of NK cells isolated from women with RSA significantly decreased after 24 and 72 h of co-culture with hAECs compared to the single culture of these cells (n = 20). This decrease was not statistically significant in the healthy group. B) CD107a expression on the surface of NK cells in the RSA group after 72 h of co-culture with hAECs significantly decreased compared to 24-h co-culture, while no significant difference was observed in the healthy group. C) CD107a expression on NK cells in the RSA group was higher compared to the healthy group. NK, natural killer cell; hAEC, human amniotic epithelial cell; RSA, recurrent spontaneous abortion. ns: $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

wells. hAECs produce large amounts of TGF-β and IL-10. pNK cells secrete large quantities of IFN-γ and also produce TGF-β and IL-10 in low concentrations.

Co-culture of hAECs with NK cells resulted in stimulation of TGF-β production. Also, the concentration of TGF-β in the supernatant of co-culture wells in both groups was significantly higher at the 1:1 ($P = 0.0064$ and $P = 0.0004$, respectively for the normal and RSA groups in 24-h co-culture, $P < 0.0001$ and $P = 0.002$, respectively for the normal and RSA groups in 72-h co-culture) and 2:1 ($P = 0.0002$, $P = 0.0004$, and $P = 0.0027$ respectively for 24-h and 72-h co-culture in the normal group and 72-h co-culture in the RSA group) cellular ratios compared to the 1:2 cellular ratio (Fig. 9A). The highest concentration of TGF-β in the study groups was associated with co-cultures of hAECs and NK cells at a 1:1 cellular ratio (Fig. 9A). There were no significant changes in the concentration of TGF-β in the study groups (Fig. 10A).

The highest concentration of IL-10 in the study groups was also related to co-cultures at the 1:1 cellular ratio (Fig. 9B). Continuing incubation for up to 72 h, the concentration of this cytokine increased significantly in the study groups (Fig. 10D). The increase in IL-10 production in the RSA group was more intense; as after 72 h of co-culture, the concentration of IL-10 in this group was significantly higher compared to the healthy group (43.93 ± 0.92 ρg/ml vs 24.68 ± 1.8 ρg/ml, $P = 0.0003$ and 25.62 ± 0.7 ρg/ml vs 17.6 ± 1.56 ρg/ml, $P = 0.0055$, respectively for 1:1 and 2:1 ratios) (Fig. 10C).

After 24 h of co-culture, the concentration of IFN-γ in the RSA group at the 1:2 (20.09 ± 0.79 ρg/ml vs 14.55 ± 0.5 ρg/ml, $P = 0.015$) and 2:1 (21.59 ± 0.1 ρg/ml vs 12.29 ± 0.06 ρg/ml, $P = 0.003$) cellular ratios was significantly higher compared to the healthy group (Fig. 10E). However, after 72 h of co-culture, the concentration of this cytokine in the RSA group was significantly lower (in all cellular ratios) compared to the healthy group

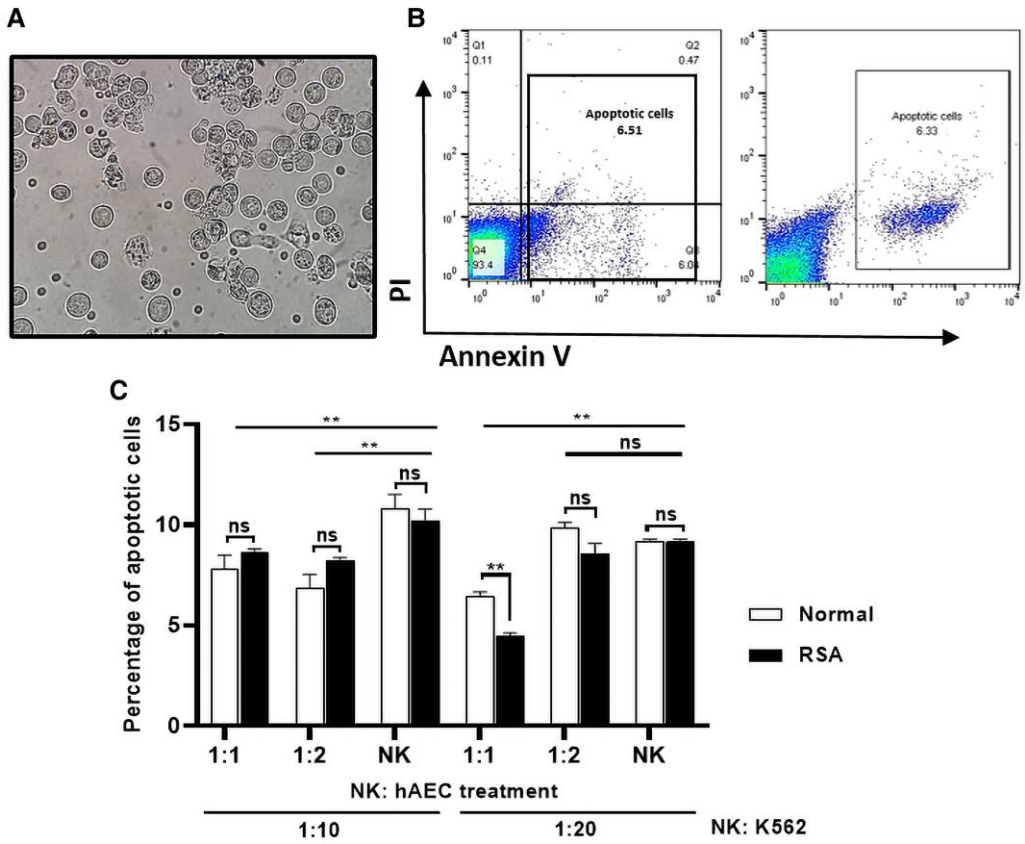

Fig. 7. Flow cytometry-based cytotoxicity assay using K562 cell line; A) Light microscope image of K562 cells; K562 cells were used as target cells in NK cytotoxicity assay performed after co-culture with hAECs. B) The strategy of gaiting for detection of early and late apoptotic cells. The Annexin V labeled cells were considered the apoptotic cell population of the K562 cell line. C) The percentage of apoptotic cells in the wells affected by co-culture-harvested NK cells significantly decreased compared to the NK cell alone (n = 2 in triplicates for each cellular ratios). However, no significant difference was observed between the study groups. NK, natural killer cell; hAEC, human amniotic epithelial cell; RSA, recurrent spontaneous abortion. ns: $P > 0.05$ and $^{*}P < 0.01$.

(8.02 ± 0.1 ρg/ml vs 12.8 ± 0.06 ρg/ml, $P = 0.0021$, 11.02 ± 0.19 ρg/ml vs 13.5 ± 0.11 ρg/ml, $P = 0.012$, and 10.3 ± 0.3 ρg/ml vs 13.64 ± 0.12 ρg/ml, $P = 0.005$, respectively for 1:2, 1:1, and 2:1 ratios). Therefore, over time, the production of IFN-γ by NK cells isolated from women with RSA affected by hAECs significantly decreased (20.09 ± 0.27 ρg/ml vs 8.02 ± 0.56 ρg/ml, $P < 0.0001$, 16.72 ± 0.24 ρg/ml vs 11.02 ± 0.49 ρg/ml, $P = 0.018$, and 21.59 ± 0.08 ρg/ml vs 10.3 ± 0.18 ρg/ml, $P < 0.0001$, respectively for 1:2, 1:1, and 2:1 ratios) (Fig. 10F).

4. Discussion

During pregnancy, hAECs and maternal NK cells interact predominantly through the secretory microenvironment. Considering the critical effect of the function and phenotype of maternal NK cells in the implantation and protection of the fetus during allogeneic pregnancy, the interaction between hAECs and NK cells will be crucial for normal pregnancy maintenance. In the present study, the presence of hAECs resulted in a decrease in the NK cell's cytotoxicity against K562 cells (target cells), an increase in the subpopulation of $CD56^{bright}CD16^{dim}$ cells, and a decline in the expression of CD107a on the surface of NK cells. Initially, the population of $CD56^{dim}CD16^{+}$ cells in women with RSA was higher than in healthy women, but after 72 h of co-culture, the percentage of these cells significantly decreased. The percentage of $CD56^{bright}CD16^{dim}$ cells in both study groups increased significantly after 72 h of co-culture. Also, the concentration of IFN-γ produced by NK cells isolated from women with RSA decreased after 72 h of incubation with hAECs. The concentration of this cytokine in the initial 24 h of co-culture was higher in the RSA group compared to the healthy group, and after 72 h, a significant decrease in the production of this cytokine resulted in a lower last concentration in the RSA group compared to healthy women.

Despite the observations indicating the successful immunomodulatory function of hAECs on the cytotoxicity and phenotype of NK cells isolated from women with RSA, the expression of CD69 and FasL markers in the co-culture wells increased significantly compared to the negative control culture of NK cells. However, the expression of FasL in NK cells isolated from healthy women decreased significantly after 72 h of co-culture with hAECs.

A notable matter about the cellular ratios in the co-culture of hAECs and NK cells is the difference in the 1:1 cellular ratio compared to other cellular ratios. The immunomodulating potency of hAECs in modulating NK cells' cytotoxicity was reduced by the increase in the ratio of NK cells to hAECs (2:1). We observed a highly stimulating effect on NK cell cytokine production in co-cultures at 1:1 cellular ratio.

An increase in the production of IL-10 and TGF-β, a decrease in the secretion of IFN-γ, and an increase in the percentage of the $CD56^{bright}$ subpopulation of NK cells indicated that the co-culture of these cells in equal ratios stimulated the shift of pNK cells to active cytokine-producing dNK cells.$CD56^{dim}CD16^{+}$ cells, as the dominant subpopulation of NK cells in peripheral blood (pNK), show an increased in peripheral and tissue NK cells in women with recurrent pregnancy loss, and their cytotoxicity is effective

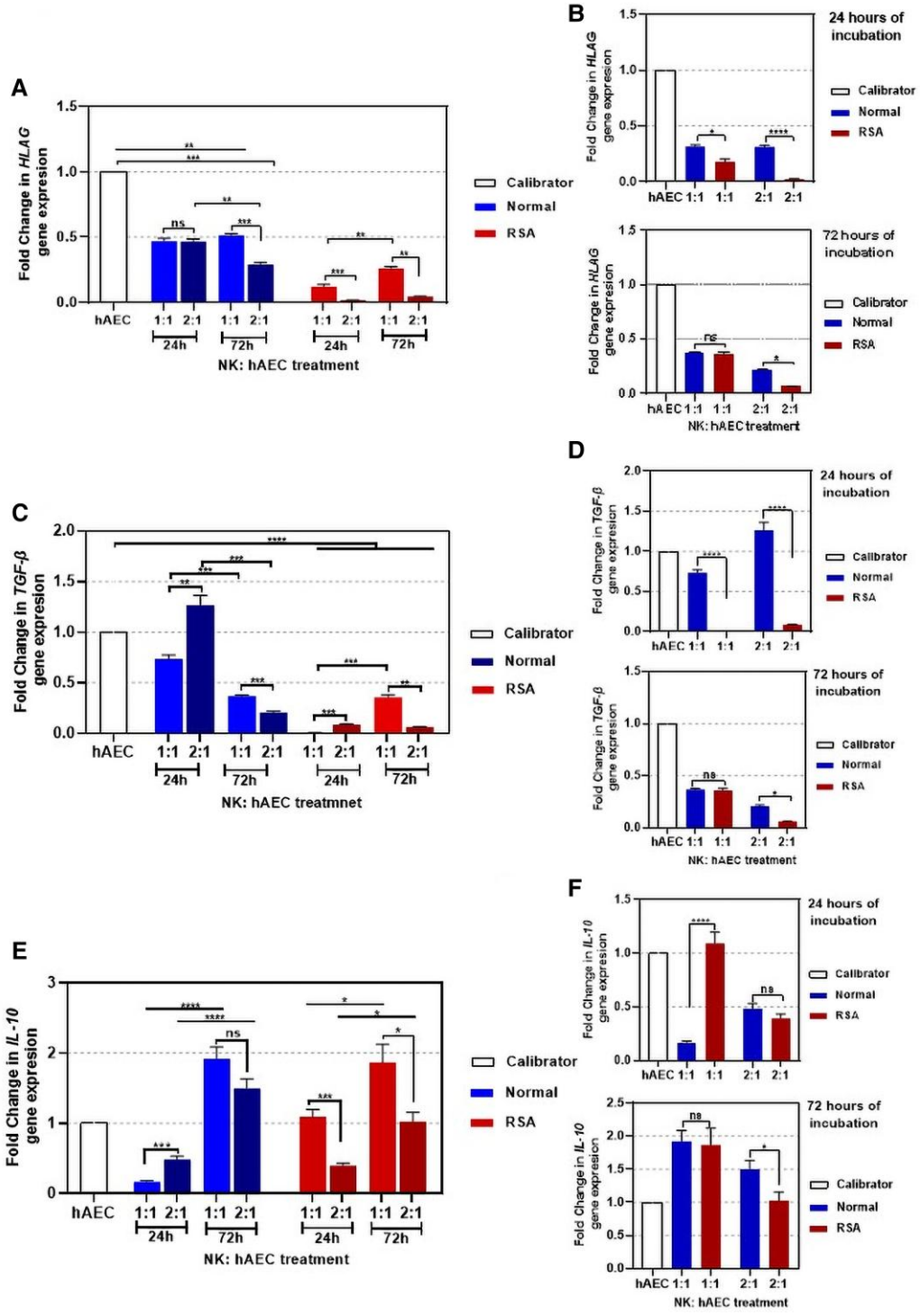

Fig. 8. The expression changes in the expression of *HLA-G*, *TGF-β*, and *IL-10* genes in hAECs after 24 and 72 h of co-culture with NK cells. The baseline of the gene expression in negative control wells of hAECs is considered a calibrator. *HLA-G* gene expression was significantly decreased at 1:1 and 2:1 cell ratios (n = 20). The decrease was statistically significant in both study groups at cell ratios 2:1 after 24 and 72 h (A). Decreased expression of *HLA-G* in the RSA group at different cell ratios after 24 h of co-culture and 72 h of co-culture was significantly higher compared to the healthy group (B). Co-culture of hAECs with pNK cells in the RSA group resulted in a significantly decreased expression of the *TGF-β* gene (n = 20). Broadly, in both groups, the *TGF-β* gene expression decreased with increasing incubation time (C). Expression of the *TGF-β* gene after 24 h of co-culture at both cell ratios and at the 2:1 cellular ratio after 72 h of incubation caused a more significant decrease in the RSA group compared to the healthy group (D). Co-culture of NK cells and hAECs at the 1:1 ratio in the RSA group and 72-h culture in the healthy group increased *IL-10* gene expression (n = 20) (E). The expression of this gene after 72 h of co-culture was significantly decreased in the RSA group compared to the healthy group (F). *HLA-G*, human leukocyte antigen G; *TGF-β*, transforming growth factor beta; *IL-10*, interleukin 10; NK, natural killer cell; hAEC, human amniotic epithelial cell; RSA, recurrent spontaneous abortion. ns: $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$.

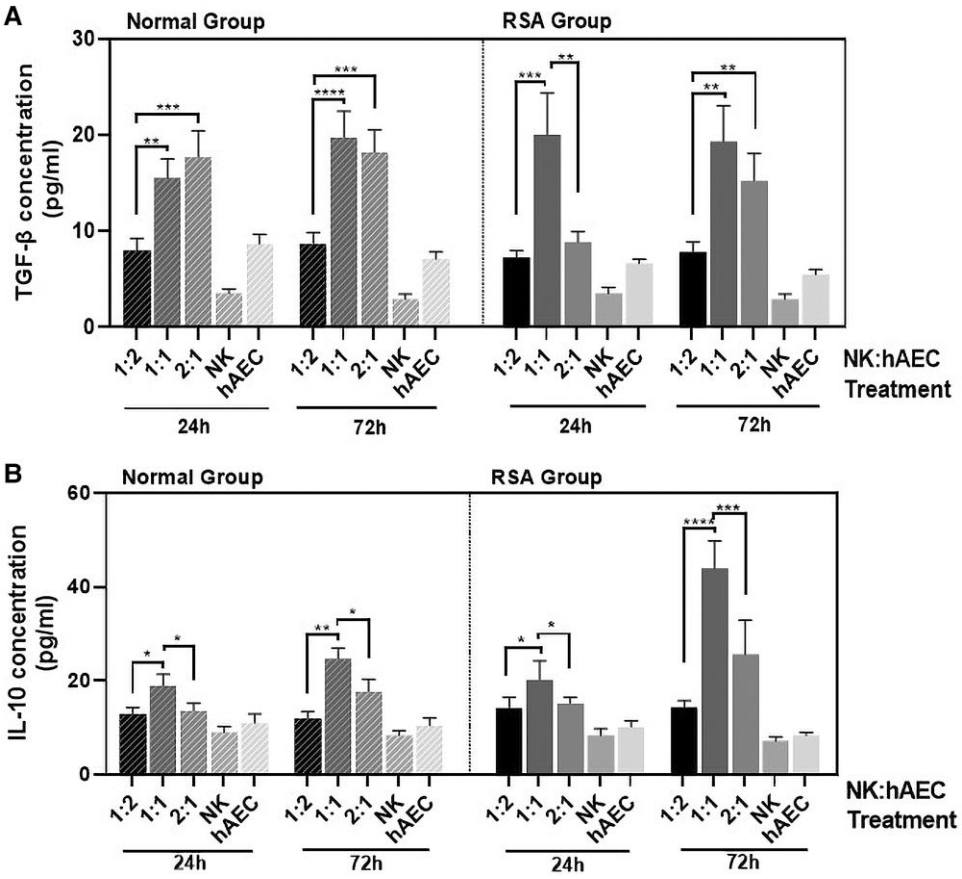

Fig. 9. Changes in the concentrations of TGF-β (A) and IL-10 (B) cytokines in the supernatant of co-culture and negative control wells at 24 and 72 h of incubation (n = 20). The highest concentration of TGF-β and IL-10 in study groups was observed at the 1:1 cellular ratio. TGF-β, transforming growth factor beta; IL-10, interleukin 10; NK, natural killer cell; hAEC, human amniotic epithelial cell; RSA, recurrent spontaneous abortion. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

in implantation failure and pregnancy loss.[35,36] It has been found that activated $CD56^{dim}CD16^{+}CD69^{+}$ NK cells are an effective subtype in preventing implantation failure and rejecting allogeneic fetuses.[37–41] CD69 is a member of the calcium-dependent lectin superfamily and is one of the first surface markers of NK cell activation.[42] CD69 has 4 ligands, one of which is galactolectin-1 (Gal-1), a β-galactoside-binding protein that plays a crucial role in regulating adaptive immune responses.[43] In healthy women, the expression and signaling of CD69 are in balance with inhibitory receptors, and its balanced activity is required for the interactions of the immune system and fetal development. Many studies have shown that implantation failure and abortion are linked to the increased expression of CD69 on the surface of NK cells.[37–41] In an in vitro study, Lee et al. indicated that the co-culture of human amniotic membrane stem cells with NK cells decreased the expression of cytotoxicity markers, including CD69 in NK cells. Gros et al. demonstrated that HLA-G-treated DCs, following co-culture with NK cells, strongly inhibited CD69 expression in NK cells compared to nontreated DCs.[44] Li et al. demonstrated that CD69 is involved in NK cell cytotoxicity against target cells like K562 cells, and suppressing the expression of CD69 is associated with decreased cytotoxicity of NK cells against target cells.[45] In another study, Khadem et al. demonstrated that CD69 and CD107a (LAMP-1) are expressed on the surface of both $CD56^{bright}$ and $CD56^{dim}$ subpopulations of NK cells in women with RSA and co-culture of these cells with hAECs resulting in a decrease the expression of CD69 and CD107a on the surface NK cells.[40] CD107a is a surface marker associated with degranulation and secretion of inflammatory cytokines by activated NK cells, and it has a lower expression on the surface of dNK cells compared to pNK cells.[46] Contrary to Khadem et al.'s findings, we did not observe any decrease in CD69 expression on the surface of NK cells. This difference is probably because Khadem et al. investigated the expression of this marker in peripheral blood mononuclear cells adjacent to hAECs, while we used a pure population of NK cells co-cultured with hAECs.[40] Accordingly, it appears that the interaction between immune cells and hAECs-conditioned media could lower CD69 expression. We also observed a decrease in CD107a expression in the co-culture of pure pNK cells with hAECs, as well as a decline in NK cell cytotoxicity against K562 cells, in which CD69 is involved.

In addition, we observed that both hAECs and allogeneic pNK cells experienced phenotypic and functional changes during their co-culture in vitro. Our study revealed that hAECs significantly decreased the expression of *HLA-G* and *TGF-β* genes in pNK cells isolated from women with RSA. The effect of pNK cells isolated from healthy women on reducing the expression of these immunomodulatory genes in hAECs was lower than that of pNK cells isolated from women with RSA. With the increase in the ratio of NK cells to hAECs, there was a marked decrease in the expression of these genes in both study groups. Co-culture in both healthy and RSA women resulted in a significant increase in the concentration of TGF-β cytokine in the supernatant of co-culture wells compared to hAECs culture. These observations indicate that the co-culture

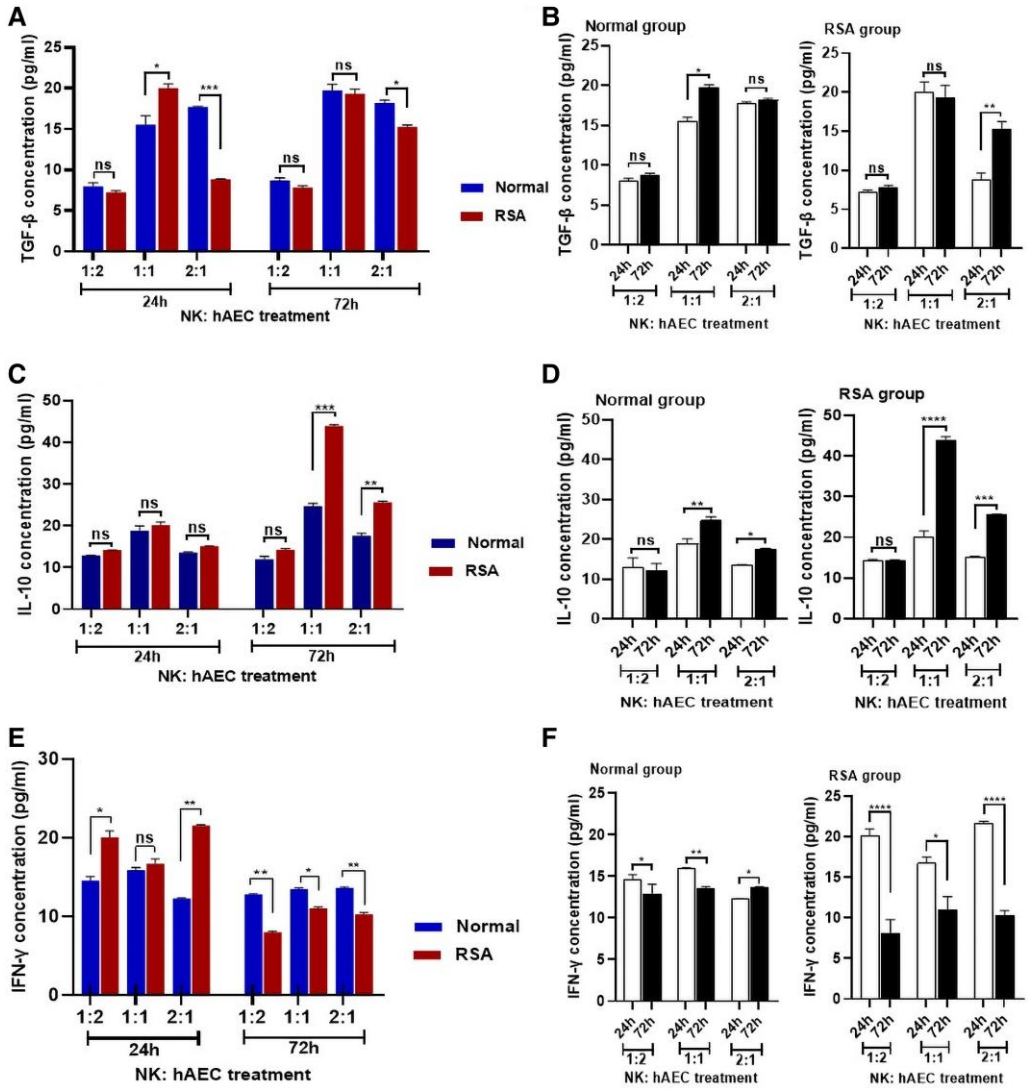

Fig. 10. The changes of the concentration of the TGF-β, IL-10, and IFN-γ cytokines in the RSA and healthy groups at 24 and 72 h. There were no meaningful differences between study groups at cellular ratios (A) or time intervals (B). In the 24-h co-culture, the IL-10 concentration did not indicate a significant difference between the study groups. After 72 h of co-culture, the IL-10 concentration in the 1:1 and 2:1 cell ratios in the RSA group significantly increased compared to the healthy group (C). IL-10 concentration over time up to 72 h revealed an increasing trend. This increase was significant at the 1:1 and 2:1 cellular ratios in both study groups (D). During the first 24 h of co-culture, the concentration of IFN-γ was increased in the RSA group, while after 72 h, its concentration significantly decreased in the RSA group compared to the healthy group (E). The concentration of IFN-γ in each of the co-culture ratios decreased significantly in the RSA group over time (n = 20) (F). TGF-β, transforming growth factor beta; IL-10, interleukin 10; IFN-γ, interferon-gamma; NK, natural killer cell; hAEC, human amniotic epithelial cell; RSA, recurrent spontaneous abortion. ns: $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

of hAECs and NK cells at a 1:1 cellular ratio resulted in an increase in TGF-β production and secretion. We observed almost the same effect regarding IL-10 gene expression by human AECs and IL-10 cytokine concentration in the co-culture supernatant. Accordingly, the equal ratio of these cells (1:1) stimulated the production of TGF-β and IL-10 cytokines in both types of cells, mainly NK cells, and this stimulating effect increased over time.

Furthermore, we found that, during the co-culture of hAECs and allogeneic pNK cells in vitro, both types of cells underwent phenotypic and functional changes. Our findings indicate that the pNK cells obtained from women with RSA had a significant decrease in the expression of *HLA-G* and *TGF-β* genes caused by hAECs. The impact of pNK cells isolated from healthy women on reducing immune modulatory genes in hAECs was less than that of pNK cells isolated from women with RSA. In both study groups, the decrease in the expression of these genes was intensified with the increase in the ratio of NK cells to hAECs. However, the concentration of TGF-β cytokine in the supernatant of co-culture wells was significantly increased due to co-culture in healthy women and women with RSA compared to hAECs culture. Based on these observations, the co-culture of hAECs and NK cells at the 1:1 cellular ratio increased the production and secretion of TGF-β. We observed almost the same effect regarding IL-10 gene expression by human AECs and IL-10 cytokine concentration in the co-culture supernatant. Accordingly, the equal ratio of these cells (1:1) stimulated the production of TGF-β and IL-10 cytokines in both types of cells, mainly NK cells, and this stimulating effect increased over time.

The TGF-β and IL-10 production is considered one of the main immunomodulatory mechanisms of hAECs. Studies have shown

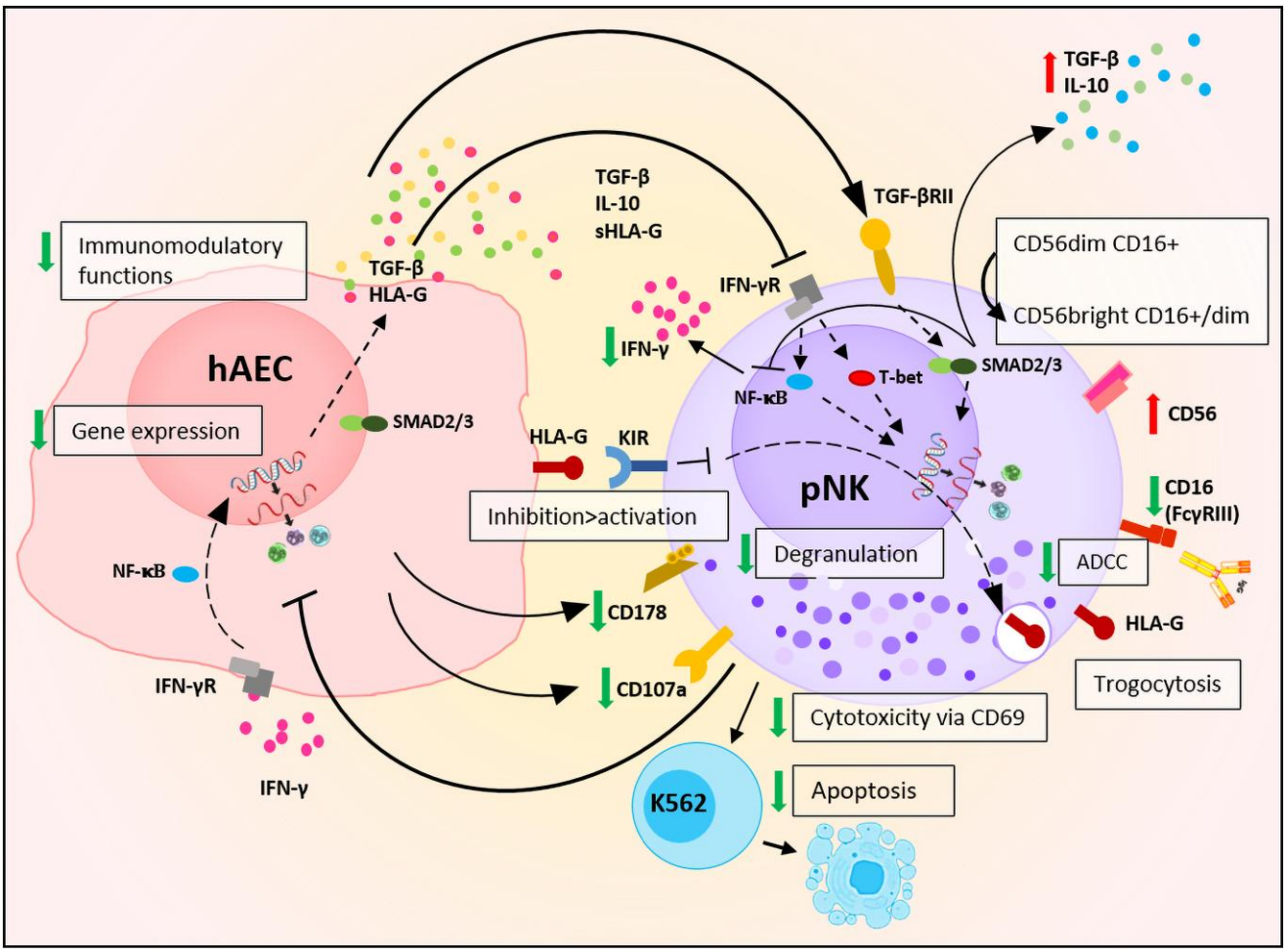

Fig. 11. Schematic view of the hypothetical signaling pathways involved in the immunomodulation of pNK cells by hAECs. NK cells are also affected by these interactions. The immunomodulatory function of hAECs decreases IFN-γ production, CD178 and CD107a expression, as well as cytotoxicity of NK cells. Additionally, CD56 expression increases, and there is an elevation in the production of TGF-β and IL-10 in these cells under the influence of hAECs. On the other hand, the interaction between hAECs and pNK cells leads to a decrease in TGF-β production and HLA-G expression in hAECs. pNK, peripheral blood NK cell; hAEC, human amniotic epithelial cell; TGF-β, transforming growth factor beta; IFN-γ, interferon-gamma; IL-10, interleukin 10; HLA-G, human leukocyte antigen-G; sHLA-G, soluble human leukocyte antigen-G; ADCC, antibody-dependent cellular cytotoxicity; KIR, killer cell immunoglobulin-like receptor; FcγRIII, Fc gamma receptor type III; TGFβRII, transforming growth factor beta receptor.

that stimulation of hAECs by lipopolysaccharide significantly increases TGF-β production after 72 h of culture.[47,48] These observations indicate the effect of inflammatory factors in stimulating TGF-β expression, demonstrating the role of cytokines in modulating severe inflammatory responses against bacterial infections in the amnion fluid and regulating the immune system at the feto-maternal interface.[49] Zhang et al., in their study on ovarian tissue regeneration and function after chemotherapy, reported that hAECs secrete TGF-β in a paracrine manner, resulting in the activation of the SMAD2 and SMAD3 signaling pathways in granulosa cells through TGF-β receptor type II.[50] Eventually, this activation leads to tissue regeneration and immune regulation in damaged tissues.

hAECs and NK cells both secrete IL-10 and TGF-β cytokines. Moreover, dNK cells in healthy women secrete higher levels of IL-10 and TGF-β cytokines compared to women with RSA,[46] and pNK cells secret these cytokines at lower levels compared to dNK cells. Resting NK cells express TGF-β receptor type II and are regulated by the TGF-β1-SMAD2, and SMAD3 signaling pathway in an autocrine manner. This signaling pathway has also been observed in dNK cells, but after the activation of these cells, the expression of the TGF-β receptor and its autocrine signaling pathway decreases.[51] According to Yu et al. findings, the activation of this signaling pathway in NK cells conflicts with the production of inflammatory cytokines such as IFN-γ,[51] and the activation of the TGF-β1-SMAD2 pathway inhibits IFN-γ production through the T-bet signaling pathway, which regulated IFN-γ production of.[52] According to the results of previous studies inhibition of the T-bet pathway can be considered as one of the immunomodulatory mechanisms of hAECs.[53,54] Therefore, in our study, the decrease in IFN-γ production by NK cells after co-culture with hAECs could be the result of the activation of the TGF-β1-SMAD2 signaling pathway and inhibition of T-bet signaling pathway. The effect of hAECs on the change of peripheral blood NK cell characterization toward dNK phenotype and function is highlighted by these findings, although additional research is needed in this area.

Consistent with our observations, Motedayyen et al. demonstrated that hAECs stimulated the production of TGF-β and IL-10 by T cells isolated from women with RSA after their in vitro co-culture. The differentiation of naive T cells into Tregs was triggered by the immunomodulatory potential of hAECs.[53,54] Furthermore, their findings revealed that hAECs decreased the

production of IFN-γ by $CD4^+$ T cells.[47] Li et al. demonstrated that hAECs decrease the production of IFN-γ by NK cells.[45] Furthermore, they have indicated, IL-10 concentration in the co-culture supernatant of amniotic membrane cells and NK cells increased compared to the negative control wells, and by removing this cytokine, the cytotoxicity function of NK cells increased again in the co-culture wells.[45] The findings of this research, including an increase in IL-10 production in co-culture wells and a significant increase in the concentration of this cytokine after 72 h of co-culture of NK cells isolated from women with RSA with hAECs, indicated the effect of IL-10 on decreasing NK cell cytotoxicity and the stimulatory effect of NK cells on the production of this cytokine by hAECs. These observations are consistent with other discussed findings about TGF-β production.

Although the immunomodulatory effects of hAECs have been considered dependent on immunosuppressive mediators.[55] Several studies have reported that the exposure of hAECs to IFN-γ increases the production of anti-inflammatory molecules, including HLA-G, PD-L1, IL-10, IL-6, IL-8, and G-CSF.[10,56]

The expression of HLA-G on the cell surface and the secretion of the soluble form of this molecule are considered the known immunomodulatory mechanisms in hAECs. Previous studies have indicated that increasing HLA-G expression is critical for a successful pregnancy, so that during pregnancy, the production of soluble HLA-G (sHLA-G) increases 2-fold to 5-fold, and the decrease in the expression of this molecule is associated with severe disorders in fertility and pregnancy.[57–59] The HLA-G interacts with 3 receptors on the surface of NK cells, including killer cell immunoglobulin-like receptor 2DL4 (KIR2DL4 or CD158d), leukocyte immunoglobulin-like receptor subfamily B1 (LILRB1, ILT2, or CD85j) and subfamily B2 (LILRB2, ILT4, or CD85d). These receptors have activating motifs as well as inhibitory motifs.[60] Previous studies have shown that pNK cells can obtain HLA-G from the transfected melanoma cell line by trogocytosis. After obtaining HLA-G, the cytotoxic activity of these cells decreases. However, this state is transient, and after the degradation of HLA-G inside the cell, the NK cell regains its cytotoxicity.[61–63] These studies in the tumor context provided new insights into the possible mechanisms of induction of HLA-G-mediated immune tolerance.

Tilbergs et al. demonstrated an HLA-G cycle of trogocytosis, endocytosis, degradation, and reacquisition in dNK cells after co-culture with extravillous trophoblasts (EVT) obtained from an autogenous pregnancy. They indicated following dNK cell activation under the influence of inflammatory cytokines or a viral infection, this cycle is affected and disrupted. This team has concluded that acquiring HLA-G by the mechanism of trogocytosis, then its endocytosis, its destruction, and finally its reacquisition on the cell surface, is transient and local and is one of the new functional characteristics of dNK cells acquired after its interaction with EVT. A similar mechanism is required to provide feto-maternal immune tolerance to the fetus and maintain dNK cells' potency to generate immune responses during normal pregnancy.[63] Despite the reports demonstrating these changes as a transient state in NK cells, Cantini et al. introduced HLA-G-receiving NK cells as $HLA\text{-}G^+$ NK-reg cells, the non-cytotoxic population of NK cells with regulatory activity.[64] Further studies in this field have shown that soluble HLA-G (sHLA-G) binds to the KIR2DL4 receptor on the surface of pNK cells, and after binding, the KIR2DL4-sHLA-G complex is endocytosed, then its signaling is carried out from the endosome.[50,53] The observation of this phenomenon in pNK cells indicates that the role of HLA-G is not limited to protecting $HLA\text{-}G^+$ cells against NK cell cytotoxicity.[65]

Therefore, according to our findings and previous studies, it can be demonstrated that hAECs, through producing HLA-G and stimulating the trogocytosis, decrease pNK cell cytotoxicity, and these changes (maybe transiently) shifted the phenotype and function of pNK cells toward dNK cells. On the other hand, in the present study, co-culture of NK cells isolated from women with RSA caused a significant decrease in the expression of *HLA-G* and *TGF-β* in hAECs. This observation may result from the trogocytosis and the HLA-G cycle defects in hAECs, which can ultimately result in feto-maternal immune tolerance disruption and miscarriage. Considering the decrease of CD107a expression on the surface of NK cells isolated from women with RSA after co-culture with hAECs, one of the possible reasons for HLA-G trogocytosis defect in NK cells is endosomes and lysosomes dysfunction in these women. Furthermore, in addition to the origin of dNK cells from peripheral blood NK cells, our results indicated that the phenotype of peripheral blood NK cells changed to dNK cells in the presence of hAECs, and this phenotype change was slighter in NK cells obtained from women with RSA. So, it can be concluded that NK cells dysfunction in RSA women associates with fractured fetal tolerance. However, the level of HLA-G protein was not determined in this study, and more detailed studies are required to confirm the dysfunction of NK cells isolated from women with RSA.

Our findings, aligned with the results of previous studies, showed that hAECs can modulate the inflammatory activity of NK cells (Fig. 11).

5. Conclusion

According to our findings, the co-culture of NK cells and hAECs diverted the phenotype and function of pNK cells toward dNK cells with low cytotoxicity and increased production of anti-inflammatory cytokines and growth factors. Also, NK cells isolated from women with RSA and healthy women showed different responses in the interaction with hAECs, probably due to the dysfunction of NK cells in these patients. On the other hand, immunomodulatory mechanisms of hAECs, including HLA-G expression, are impaired under the impact of NK cells isolated from women with RSA. However, extensive research is required to assess NK cell defects and their mutual interaction with hAECs in RSA.

Author contributions

Fatemeh Rezayat: conception and design, performing experiments, collection and analysis of data, provision of study materials, manuscript writing, and manuscript final approval. Nafiseh Esmaeil: conception and design, Performing experiments, data collection, data analysis, and interpretation, financial support, manuscript writing, and manuscript final approval. Abbas Rezaei: provision of study patients, manuscript writing, and final approval of manuscript. Parvaneh Nikpour: data analysis and interpretation and manuscript writing, and manuscript final approval. Awat Feizi: statistical analysis of data and manuscript final approval.

Supplementary material

Supplementary material is available at *Journal of Leukocyte Biology* online.

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Consent for publication. All authors hereby provide consent for the publication of the manuscript detailed above.

Data availability

The data are available from the corresponding author upon request.

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