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Interactions of NK Cells and Trophoblast Cells. Methodological Aspects

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NK cells present in different organs differ by their functional characteristics, in particular, proliferative activity. For studying tissue-resident NK cells, tissue-specific microenvironment should be reproduced. In case of decidual NK cells, this microenvironment is created by trophoblast cells. We developed a method for evaluation of proliferative activity of peripheral blood NK cells in the presence of trophoblast cells. Proliferative activity of peripheral blood NK cells was evaluated by the expression of protein Ki-67 after culturing with JEG-3 trophoblast cells. This method allows evaluating the functional state of NK cells in microenvironment specific for the decidua.

Key Words: *NK cells; trophoblast; proliferation; Ki-67; JEG-3*

The main function of natural killer cells (NK cells), effectors of innate immunity, is contact cytolysis of virus-infected and tumor cells. In addition, NK cells are the source of cytokines affecting other cells of the microenvironment [16,19]. Until now, the origin of NK cells remains unclear. According to modern views, NK cells are type 1 innate immunity lymphoid cells (ILC); they can be subdivided into conventional and non-conventional NK cells. The latter include NK cells of the thymus, liver, lungs, skin, uterus, kidneys, pancreas, and salivary glands [12]. NK cells in these organs differ by their phenotype [17] and functional state [9]. It is believed that cells of the microenvironment can determine differences in the phenotype and the degree of NK activation. Functional status of NK cells can be assessed by different methods, mainly by measuring their cytotoxic activity. Evaluation of cytotoxic activity of NK cells against K562 cells is most often used [1]. Degranulation of NK cells can be assessed by an indirect method based on detection of lysosomal glycoprotein CD107a is [1,3]. A modified method for measuring cytotoxic activity of NK cells is based on assessment of apoptosis of the target cells by the release of intracellular caspases [1]. Another important functional characteristic of NK cells is their proliferation. *In situ* proliferation can determine the formation and replenishment of the pool of resident NK cells. A widely used method for evaluation of cell proliferation is based on the decrease of fluorescence intensity of cells pre-treated with vital dye carboxyfluorescein succinimidyl ester (CFSE): the peaks of CFSE fluorescence correspond to the number of cells divisions [18]. Proliferation can also be assessed by the expression of nuclear Ki-67 protein; the intensity of its expression correlates with cell progression through the cell cycle [23]. These methods are widely used for evaluation of NK-cell activity.

Studies of the interaction of NK cells with other immunocompetent and tissue-specific cells are essential for better understanding of not only common cytotoxic effects, but also other physiological and pathophysiological processes. From the viewpoint of reproductive biology and medicine, the interaction of NK cells and trophoblast cells is of particular interest. Functional characteristics of non-classical NK cells, *e.g.*, uterine NK cells, differ from the corresponding characteristics of peripheral blood NK cells [2]. NK cells are present in the endometrium of non-pregnant

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women. During pregnancy, decidualization of the endometrium is accompanied by accumulation of NK cells in the decidua [4]. Trophoblast cells participate in remodeling of the spiral arteries [5] and can interact with decidual NK cells located along the spiral arteries, which enables mutual modulating effects. The most probable is the interaction of decidual NK cells and extravillious trophoblast cells [11,26]. Trophoblast cells secrete various cytokines [5,26] capable of modulating functional state of NK cells in the decidua. To study the peculiarities of the functional state of decidual NK cells, it is necessary to reproduce tissue-specific conditions, including cellular and cytokine microenvironment created primarily by trophoblast cells. As the possible mechanism of NK cell accumulation in the decidua is their migration from the peripheral blood along the concentration gradient of chemokines secreted by trophoblast cells [22], peripheral blood NK cells can be used to study tissue-specific interactions in the decidua. In view of possible contacts of NK cells and trophoblast cells in women during pregnancy, NK cells derived from non-pregnant women should be used to model *in vitro* interactions occurring *in vivo* in the decidua.

Our aim was to develop a method for evaluation of proliferative activity of peripheral blood NK cells in the presence of trophoblast cells.

MATERIALS AND METHODS

The study included 61 healthy non-pregnant women aged from 19 to 23 years (mean 20.3±0.2 years). Exclusion criteria were type 1 diabetes mellitus, hydramnios, oligohydramnios, urogenital infection, acute or exacerbating chronic infection, hypertension, and other blood circulatory system diseases.

Peripheral blood samples were obtained through venipuncture of the ulnar veins using a 16G needle and collected in standard evacuated tubes with anticoagulant heparin (Vacuette; Greiner Bio-One). The study was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki). All patients gave informed consent for participation in the study. The study was approved by the Ethics Committee of D.O. Ott Research Institute of Obstetrics, Gynecology, and Reproductology.

We used JEG-3 trophoblast cells obtained from the cell culture bank (ATCC). These cells reproduce all the major morphological, phenotypic, and functional characteristics of first-trimester extravillous trophoblast cells. JEG-3 cells were cultured in a humid atmosphere at 37°C and 5% CO_2 in DMEM containing 10% inactivated fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine, 10 mM sodium pyruvate, and 1% non-essential amino acids (Sigma-Aldrich).

Peripheral blood mononuclear cells were isolated under sterile conditions by centrifugation in Histopaque-1077 density gradient (Sigma-Aldrich). The proportion of viable mononuclear cells was estimated by staining with red fluoresced dye propidium iodide (PI) in a final concentration of 0.01 mg/ml (Sigma-Aldrich). PI enters the cell only through damaged membranes and binds to nuclear DNA, which makes possible detection of dead cells by by flow cytofluorometry [8]. The number of viable cells was 97.6±2.2%.

One day before the experiment, JEG-3 cells were subcultured, an aliquot of the cells suspension was resuspended in a culture medium, placed in wells of a 96-well flat-bottom plate (BD) in a concentration of 25,000 cells per well in 100 μl of culture medium, and cultured in a humid atmosphere with 5% CO_2 at 37°C for 24 h to confluence. Then, the culture medium was removed from the JEG-3 cell monolayer plate and mononuclear cells were added (100,000 cells per well in 100 μl culture medium). Wells without JEG-3 cell monolayer served as the control. In some wells, recombinant IL-2 (Roncoleukin, Biotech) was also added in a concentration of 200 U/ml. The cells were cultured in a humid atmosphere with 5% CO_2 at 37°C for 72 h. Then, 100 μl fresh culture medium and 200 U/ml recombinant IL-2 were added and the wells were cultured for the next 72 h. In 6 days, the proportion of viable peripheral blood mononuclear cells was 93.0±2.7%. The cells were harvested by adding 0.5 mM EDTA for 10 min. The viability of mononuclear cells after treatment with EDTA was 84.0±2.8%. Then, the cells were fixed and permeabilized using the commercial Cytofix/Cytoperm kit (BD Biosciences) and treated with monoclonal antibodies to CD45, CD56, CD3, and Ki-67 labeled with fluorescent labels according to the protocol recommended by the manufacturer (BD). Fluorescence of NK cells was analyzed on a FACSCanto II flow cytofluorometer (BD). For gating of NK cells, the following strategy was used: a region P1 containing mononuclear cells was selected on the direct (FSC)-side (SSC) light scattering plot. Then, events from this region were projected to a graph with the coordinates of CD45-FSC (cells with the $CD45^+$ phenotype). Then, events from the $CD45^+$ region were projected to a graph with coordinates of CD3-FSC (cells with phenotype $CD3^-$). Then, events from $CD45^+$ region were projected to a graph with coordinates of CD56-Ki-67 (cells with $CD56^+$ и $Ki\text{-}67^+$ phenotypes). Thus, we analyzed the relative number of NK cells ($CD45^+CD3^-CD56^+$) among all mononuclear cells and fluorescence intensity of NK cells treated with FITC-labeled antibodies to Ki-67 (Fig. 1). The boundaries were determined based on the previous measurement of fluorescence of cells treated with isotypic antibodies to Ki-67 (Fig. 1).

Statistical analysis was carried out by the Wilcoxon's test using Statistica software, the differences were significant at $p<0.01$ and $p<0.001$. The results are presented as median (Me) and the upper and lower quartiles (Q_1; Q_3).

RESULTS

Prior to culturing, the relative number of peripheral blood NK cells in the mononuclear fraction was 11.8 (8.8; 5.4)%. The analysis of proliferation of NK cells after isolation from the mononuclear fraction prior to culturing revealed no Ki-67 expression. After 6-day culturing both in the presence of trophoblast cells (0.8 (0.4; 1.6)%) and without them (0.4 (0.2; 0.7)%), the relative number of NK cells was lower ($p<0.001$) than before culturing (Fig. 2). The number of NK cells after culturing in the presence and absence of trophoblast cells was similar. In the presence of JFG-3 trophoblast cells, the intensity of the expression of Ki-67 proliferation marker by NK-cell was lower than in the absence of trophoblast cells (Table 1, Fig. 2).

After culturing of NK cells in the presence of IL-2, their number was higher (16.5 (13.7; 22.9)%) than before culturing ($p<0.001$). Moreover, their number and intensity of proliferation were higher than in the absence of IL-2 and trophoblast cells (Table 1, Fig. 2).

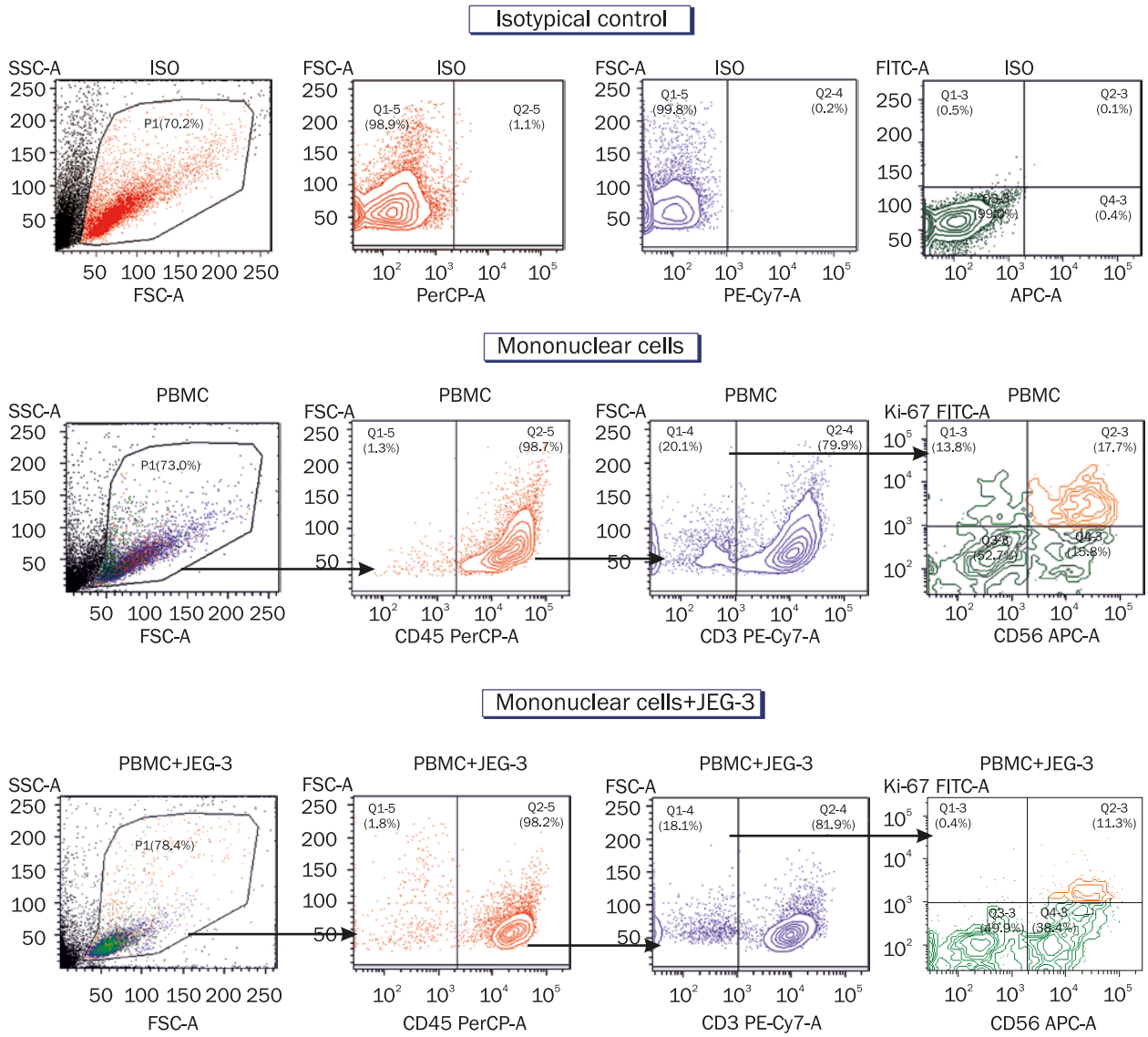

Fig. 1. Proliferative activity of peripheral blood NK cells after 6-day culturing. Isotypic control: 2D histogram in coordinates FSC-SSC, PerCP-FSC, PE-Cy7-FSC, and APC-FITC. Mononuclear cells after culturing separately and with JEG-3 trophoblast cells treated with antibodies to CD45, CD3, CD56, Ki-67: 2D histogram of mononuclear distribution in coordinates FSC-SSC; CD45 PerCP-FSC; CD3 PE-Cy7-FSC; CD56 APC-Ki-67 FITC.

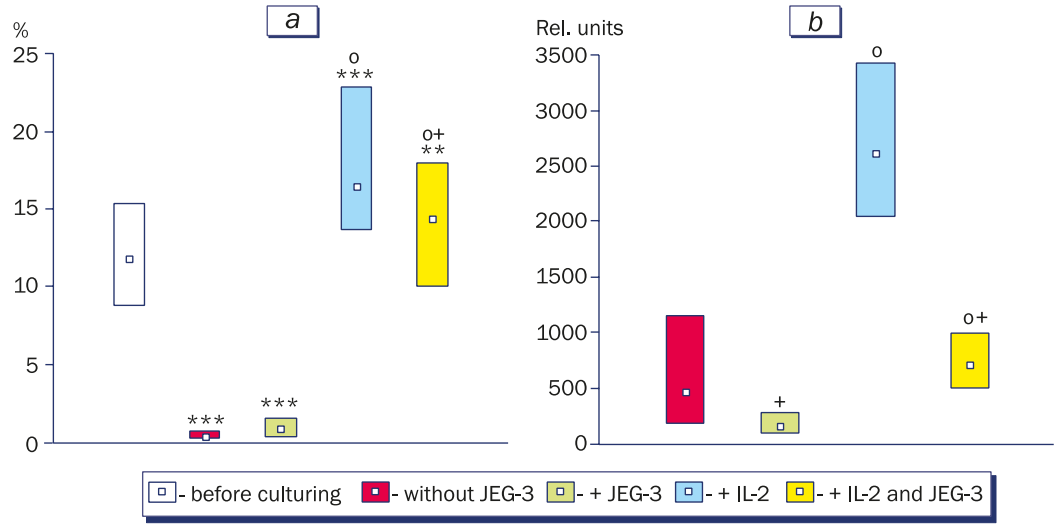

Fig. 2. Quantitative indicators of proliferation of peripheral blood NK cells. *a*) Relative number of NK cells; *b*) intensity of Ki-67 expression by NK cells. ***p*<0.01; ****p*<0.001 in comparison with the corresponding value before culturing; +*p*<0.001 in comparison with culturing without JEG-3 trophoblast cells; °*p*<0.001 in comparison with culturing without IL-2.

After culturing of NK cells in the presence of IL-2 and trophoblast cells, their number was also higher (14.4 (10.0; 18.0)%) than before culturing ($p<0.001$) and after culturing in the presence of only trophoblast cells (Table 1; Fig. 2). The intensity of Ki-67 expression by NK cells after culturing in the presence of IL-2 and trophoblast cells was higher than after culturing in the presence of only trophoblast cells (Table 1, Fig. 2).

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Functional state of NK cells depends on the microenvironment in various tissues [12]. The functional state of uterine NK cells is modulated by interaction with trophoblast cells, which contributes to physiological development of pregnancy [26]. Taking into account this mutual influence, we can assume that trophoblast cells can regulate proliferative activity of NK cells and, hence, manifestation of functional activity of NK cells at the uteroplacental interface. We developed and tested a new method for assessing the effect of trophoblast cells on functional activity of NK cells. During testing of this method, we found that the relative content of NK cells significantly decreased during culturing without inducers and in the presence of trophoblast cells without additional inducers. These data indicate the need of using additional inducers for the culturing of NK cells for 6 days, including culturing in the presence of trophoblast cells. It was previously shown that JEG-3 trophoblast cells secrete IL-15 [24]. In the presence of picomolar concentrations of IL-15, NK cells retain their viability for more than 7 days due to expression of the anti-apoptotic factor Bcl-2 [7]. It appears that in our model, the secretory products of trophoblast cells do not appreciably maintain viability of NK cells.

It has been previously reported that IL-2 administered intravenously to patients with advanced cancer and administered subcutaneously to patients with chronic "graft—versus—host" reaction stimulated proliferative activity of peripheral blood NK cells [14]. The use of IL-2 in *in vitro* models led to an increase in the intensity of NK cell proliferation and increased secretion of IFNγ after activation with PMA and ionomycin [10]. In our experiments we, similar to other investigators [15,25], used IL-2 in a concentration of 200 U/ml as an additional inducer of proliferative activity. In this concentration [15,25], and in lower concentrations [10], IL-2 increased cytotoxic activity of NK cells and modulated their proliferation [10].

The observed increase in the number of NK cells after incubation with IL-2, as well as in the presence of IL-2 and trophoblast cells, *vs.* the initial number of NK cells and the number of NK cells after incubation without IL-2, attested to stimulation of proliferation by IL-2, which is consistent with published report [10]. This assumption is supported by the data on enhanced expression of Ki-67 by NK cells in the presence of IL-2 both with and without trophoblast cells in comparison with the corresponding incubation conditions, but without IL-2. At the same time, proliferative activity of NK cells cultured in the presence of trophoblast cells, both in the presence or absence of IL-2, was reduced in comparison with proliferation of NK cells incubated without trophoblast cells. The possibility of initiating apoptosis after stimulation of peripheral blood NK cells with IL-2 in combination with IL-12 or

TABLE 1. Proliferation of Peripheral Blood NK Cells

Parameter	After culturing without trophoblast cells	After culturing with trophoblast cells	After culturing with IL-2	After culturing with IL-2 and trophoblast cells
Relative number of NK cells, %	0.4 (0.2; 0.7)	0.8 (0.4; 1.6)	16.5 (13.7; 22.9)$^{+}$	14.4 (10.0; 18.0)$^{*+}$
Intensity of Ki-67 cell fluorescence, rel. units	464 (179; 1150)	162 (94; 286)*	2617 (2049; 3428)$^{+}$	714 (509; 990)$^{*+}$

Note. $p<0.001$ in comparison with *culturing without trophoblast cells; $^{+}$culturing without IL-2.

with IL-15 in combination with IL-12 was previously reported [21]. Expression of IL-12p70, an active heterodimeric form of IL-12 [20], was demonstrated for cells of both villous and extravillous trophoblast [20], which suggests that IL-12 produced by trophoblast cells can act as the agent stimulating NK cell apoptosis in our model. JEG-3 trophoblast cells were shown to secrete TGFβ [13,27]. It was previously demonstrated that the presence of TGFβ1 in the *in vitro* culture of NK cells suppressed production of IFNγ, TNFα, and granulocyte-macrophage CSF by peripheral blood NK cells and inhibited DNA synthesis by NK cells (*i.e.*, their proliferation) [6]. These changes attest to possible role of secretory products of trophoblast cells in suppression of NK cell proliferation in our model.

Thus, the method for assessment of proliferation of peripheral blood NK cell allows evaluating the functional state of NK cells in the microenvironment of trophoblast cells. Using this model system, we found that trophoblast cells modulate the functional state of peripheral blood NK cells and maintain their number at a certain level via suppression of their proliferation. This effect can be mediated by cytokines IL-15, IL-12, and TGFβ secreted by trophoblast cells. The proposed model can be used for evaluation of the interaction of trophoblast with NK cells during pregnancy.

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