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Cytotoxic Activity of Peripheral Blood NK Cells towards Trophoblast Cells during Pregnancy

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We evaluated cytotoxic activity of peripheral blood NK cells towards trophoblast cells. NK cells either isolated or in the composition of mononuclear cell fraction, caused death of trophoblast cells. In women with physiological pregnancy, the cytotoxic effect of NK cells present in mononuclear cell fraction preincubated with IL-2 was lower than in nonpregnant women, who had never been pregnant previously, and in fertile women. Cytotoxic activity of isolated NK cells preincubated with IL-2 in fertile women was lower than in nonpregnant women, who had never been pregnant previously.

Key Words: *NK cells; cytotoxic activity; trophoblast; proliferation; JEG-3*

NK cells exhibit cytotoxic activity towards altered (transformed, virus-infected, and stressed) cells of the body. NK cells are present in most organs and tissues, including bone marrow, lymph nodes, blood, skin, intestine, liver, lungs, and uterus [6,29]. Uterine NK cells represent a special cell population; the number of these cells depends on the phase of the menstrual cycle [30]. During the first trimester of pregnancy, the number of NK cells in the endometrium increases and ranges from 50 to 90% of lymphoid cells [11]. During physiological pregnancy, NK cells have a regulatory function: they create optimal conditions for blastocyst invasion, participate in remodeling of spiral arteries of the uterus, and establish normal uteroplacental blood flow [30]. The use of NK cells isolated from the decidua in experimental studies is associated with collection of biopsy material (local tissue sample), that is possible only in the absence of pregnancy, provides a small amount of material, and is associated with cell loss during the isolation procedure [26]. The use of the peripheral blood is more preferable, as it is less invasive than biopsy and allows collecting samples from pregnant women as well. According to published reports, the functional status of peripheral blood NK cells reflects the functional status of uterine NK cells [19]. There is no consensus on the origin of individual populations of uterine NK cells during pregnancy. One of the main processes that can underlie the replenishment of the NK cell population in the decidua is migration of NK cells from maternal peripheral blood [1]. In this context, analysis of changes in functional characteristics of peripheral blood NK cells during pregnancy is of crucial importance.

The most important function of NK cells is contact cytolysis of target cells (virus-infected or tumor cells). At present, several laboratory methods for measuring cytotoxic activity of NK cells are known. One of these is measuring of the intensity of K562 cell death after their incubation with NK cells. The target K562 cells are usually labeled with fluorescent dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) that forms stable covalent bond with intracellular proteins and does not affect cell viability in concentrations <5 μM. Along with measuring cytotoxic activity of NK cells by target cell lysis, another method based on the detection of degranulation marker CD107a is used [2]. This glycoprotein is bound to lysosomal membranes are appears on the surface membrane of NK cells during cytotoxic reaction associated with the release of granzymes and

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perforin [5]. Despite the correlation between CD107a expression and cytotoxicity of NK cell, analysis of the intensity of CD107a expression indirectly reflects functional activity of NK cells. Cytotoxic activity of NK cells can be evaluated by activation of intracellular caspases [16].

It should be noted that all these methods characterize the cytotoxic function out of their specific pathophysiological context and ignore possible role of target cells that can modulate functional activity of NK cells. In particular, the described methods for evaluating the cytotoxic activity of NK cells do not take into account their specific functional interactions during pregnancy. Direct interaction of trophoblast cells and NK cells is an essential process accompanying physiological pregnancy. According to published data, binding of HLA-G locus molecule expressed on trophoblast cells with KIR2DL4 receptor of NK cells stimulates secretion of IFNγ by NK cells with parallel inhibition of their cytotoxic activity towards trophoblast cells [12]. NK cells have a significant impact on placentation, *e.g.* via production of cytokines IL-8 and IP-10 that promote trophoblast invasion [15]. However, the interaction between trophoblast cells and NK cells during pregnancy is poorly studied. The existing methods for assessing cytotoxic activity of NK cells do not allow *in vitro* simulation of these cell—cell interactions.

Our aim was to evaluate cytotoxic activity of peripheral blood NK cells towards trophoblast cells.

MATERIALS AND METHODS

Patients. The study included healthy nonpregnant women without previous pregnancies (group 1, control; age 19-23 years; mean 20.3±0.2), healthy nonpregnant women with one or more physiological full-term pregnancies (fertile women; group 2; age 28-33 years, mean 30.4±0.8 years), and women with uncomplicated (physiological) pregnancy at 6-7 weeks gestation (group 3; age 18-38 years, mean 29.3±0.9 years). A special control group consisted of healthy men (group 4; age 20-35 years, mean 24.3±3.2 years). Cytotoxic activity of NK cells of the mononuclear fraction was measured in samples from 115 women (N=50 in group 1; N=40 in group 2; N=25 in group 3), and 15 men (group 4). Cytotoxic activity of isolated NK cells was measured in samples from 34 women (N=13 in group 1; N=11 in group 2; N=10 in group 3). Exclusion criteria were diabetes mellitus type 1, pelvic inflammatory disease, acute infections or exacerbation of chronic diseases, hypertension and other cardiovascular diseases.

The peripheral blood was taken from the cubital vein with a 16G needle into vacutainers with an 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 protocol was approved by local ethical committee.

Cell cultures. We used JEG-3 trophoblast cells that reproduce all major morphological, phenotypic, and functional characteristics of first-trimester extravillous trophoblast cells. The cells were cultured in DMEM supplemented with 10% inactivated fetal calf serum, 100 μg/ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1% non-essential amino acids (Sigma-Aldrich). The cells were grown in a humid atmosphere at 37°C and 5% CO_2 and subcultured every 3-4 days; to this end, the monolayer was disintegrated with 1:1 mixture of trypsin and Versene (BioloT) for 5 min.

Isolation of peripheral blood mononuclears. Peripheral blood mononuclear cells were isolated routinely by centrifugation in Histopaque-1077 density gradient (Sigma-Aldrich) and then cultured for 4 days in the presence of recombinant IL-2 (200 U/ml; Roncoleukin, BIOTECH) or without it.

Isolation of NK cells from the mononuclear fraction of the peripheral blood. The population of NK cells was isolated from the peripheral blood mononuclear fraction on a FACSAria III flow cell sorter (BD) using antibodies to CD45, CD3, CD56 (BD) by $CD45^+CD3^-CD56^+$ phenotype (Fig. 1) by the Purity protocol and using a nozzle with a diameter of 85 μ. The fraction of viable NK cells assessed after treatment with 7AAD (BioLegend) was 91.6±2.9%. Then, NK cells were cultured for 4 days in the presence of recombinant IL-2 (200 U/ml) or without it.

Evaluation of cytotoxic activity of peripheral blood NK cells towards JEG-3 trophoblast cells. In 24 h prior to the experiment, trophoblast cell monolayer was disintegrated with trypsin—Versene mixture (1:1), a half of the resulting cell suspension was transferred to a flask for culturing adhesion cultures with complete culture medium containing 1% HEPES and 10% fetal calf serum. On the next day, the trophoblast cell monolayer was disintegrated, trophoblast cells were treated with 4 μM CFSE solution and placed in a 96-well plate (6×10^5 cells/ml). Peripheral blood mononuclear cells were added to the wells in 10:1 effector:target cell ratio. In some plates, NK cells isolated from mononuclear cell fraction were added to trophoblast cells at a ratio of 5:1 (due to the limited amount of cells after cell sorting). We have previously assessed the effect of effector:target cell ratio on cytotoxic activity of NK cells. No differences between trophoblast cell death in the presence of isolated NK cells in ratios of 5:1 and 10:1 were revealed. The plates were then centrifuged for 3 min at 100g and the cells

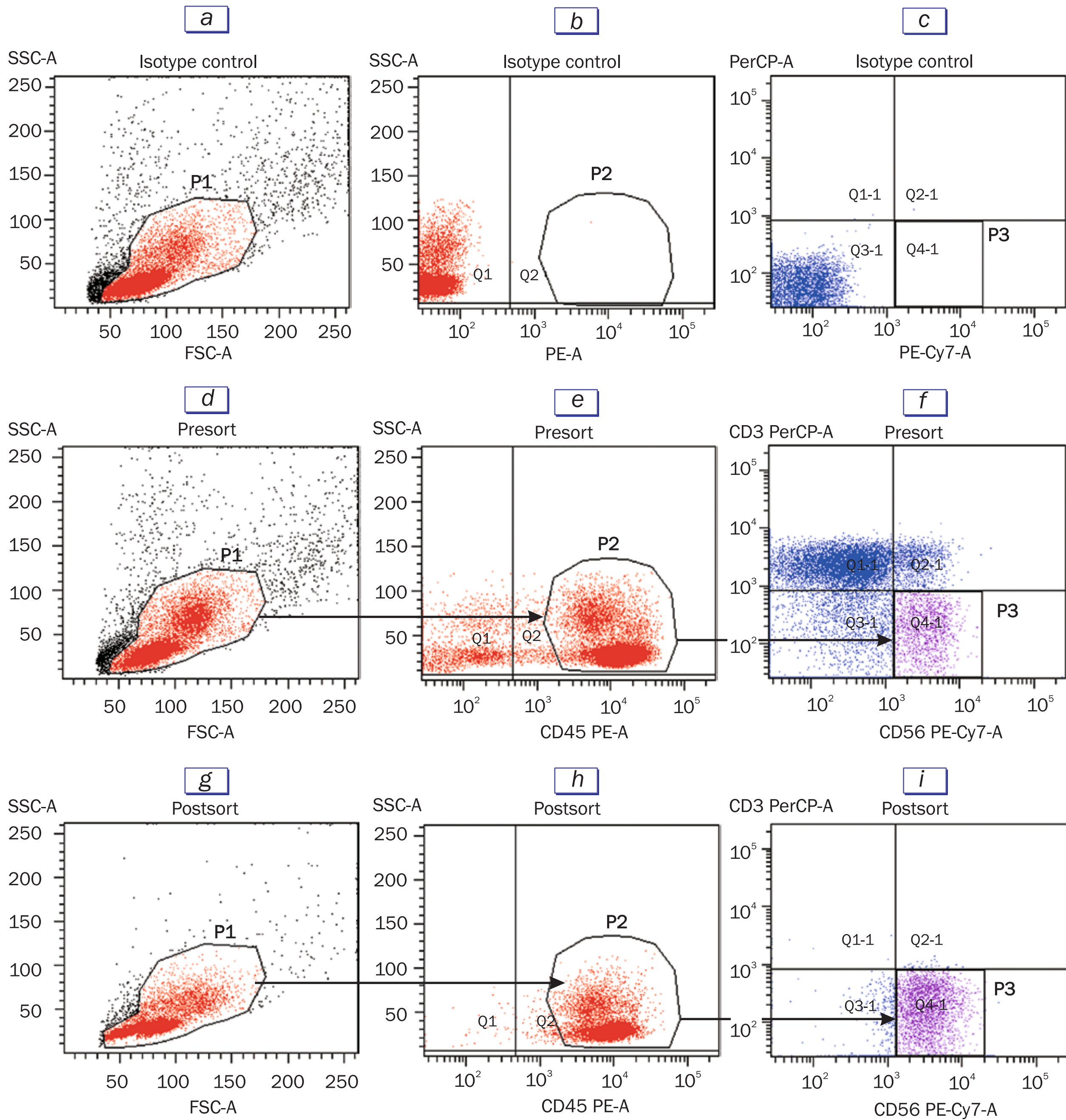

Fig. 1. Strategy of NK cell gating used in sorting of mononuclear cells.

a, *d*, *g*) FSC-SSC coordinates: gait P1 contains lymphocytes and monocytes; *b*) SSC-PE coordinates: quadrants Q1 contains lymphocytes and monocytes; *c*) PerCP-PE-Cy7 coordinates; *e*, *h*) SSC-CD45 coordinates: gait P2 contains lymphocytes and monocytes; *f*, *i*) CD3 PerCP-CD56 PE-Cy7 coordinates: gait P3 contains NK cells. The following gating strategy was used to sort the cells: fluorescence boundaries were established based on the measurement of a sample treated with isotype control (Isotype). The target cells for sorting (Presort) were isolated based on the measurement of a sample treated with antibodies. Each round of NK cell isolation in the sorter was accompanied by evaluation of the purity of the resulting cell suspension (Postsort). The purity of isolation in all cases was no less than 99±1%.

were incubated for 4 h. Some of trophoblast cells were incubated in the culture medium without mononuclear cells to determine the basal level of trophoblast cell death. Then, the content of viable and unviable trophoblast cells was assessed by staining with PI in a final concentration of 0.01 mg/ml (Sigma-Aldrich) (Fig. 2). Some trophoblast cells were not treated with CFSE solution and PI and used as a negative control in the evaluation of cell fluorescence.

Statistical analysis. The data were processed using Statistica 10.0 software. The Mann—Whitney *U* test and Wilcoxon's rank test were used for compari-

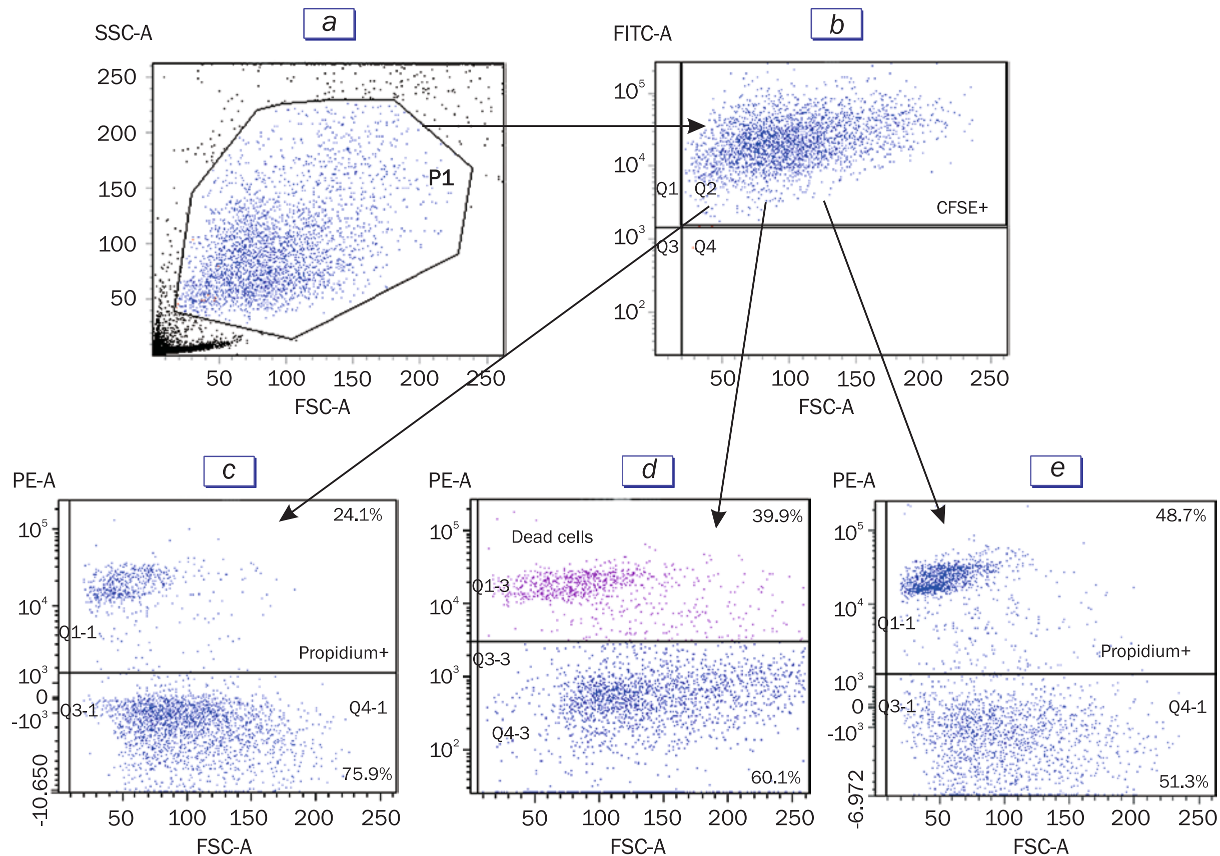

Fig. 2. Evaluation of cytotoxic activity of peripheral blood NK cells towards JEG-3 trophoblast cells. *a*, *b*) JEG-3 cells treated with CFSE; *c*) JEG-3 cells treated with PI after incubation in complete growth medium DMEM (background death); *d*) JEG-3 cells treated with PI after incubation with peripheral blood mononuclear cell fraction containing NK cells; *e*) JEG-3 cells treated with PI after incubation with peripheral blood NK cells isolated from mononuclear cell fraction.

son of the obtained results. The differences were significant at $p<0.05$.

RESULTS

NK cells either isolated or in the composition of mononuclear cell fraction, increased death of trophoblast cells (Figs. 3, 4). In all examined groups, the cytotoxic effect of NK cells towards trophoblast cells in both cases was more potent after preincubation with IL-2 in comparison with preincubation without IL-2 (Figs. 3, 4). The increase in cytotoxicity of peripheral blood NK cells in the presence of an activator (IL-2) in the model system suggests that this experimental scheme can be applied for evaluation of changes in the functional status of NK cells. It was found that the cytotoxic effect of NK cells on trophoblast cells, both in the presence of IL-2 and without it, did not differ in groups of healthy nonpregnant women and healthy men, which attested to similar basic functional status of NK cells in these groups.

When incubated without IL-2, isolated NK cells and NK cells in the fraction of mononuclear cells produced similar cytotoxic effect on trophoblast cells in all examined groups (Figs. 3, 4). It was previously shown that JEG-3 trophoblast cells secrete IL-15 [28]; secretion of this cytokine was also revealed in the placenta [4]. IL-15 increases the cytotoxic activity of NK cells and is used in antitumor therapy [22]. However, NK cells preincubated with IL-15 and then incubated with conditioned media of Sw.71 line trophoblast cells showed a decrease in the expression of perforin and granzyme B mRNA [20]. Conditioned media of trophoblast cells inhibited the expression of T-bet and Eomes in NK cells preincubated with IL-15[20]. A similar effect was demonstrated on the primary culture of trophoblast cells co-cultured with decidual NK cells. Trophoblast cells suppressed the expression of T-bet and Helios transcription factors in NK cells, which can result in a decrease in IFNγ production and the acquisition of the regulatory phenotype by NK cells [10]. It can be hypothesized that IL-15 secreted by JEG-3 cells maintains the cytotoxic activity of NK cells towards trophoblast cells at a certain level. However, contact cell—cell interactions lead to suppression of the expression of some transcription factors

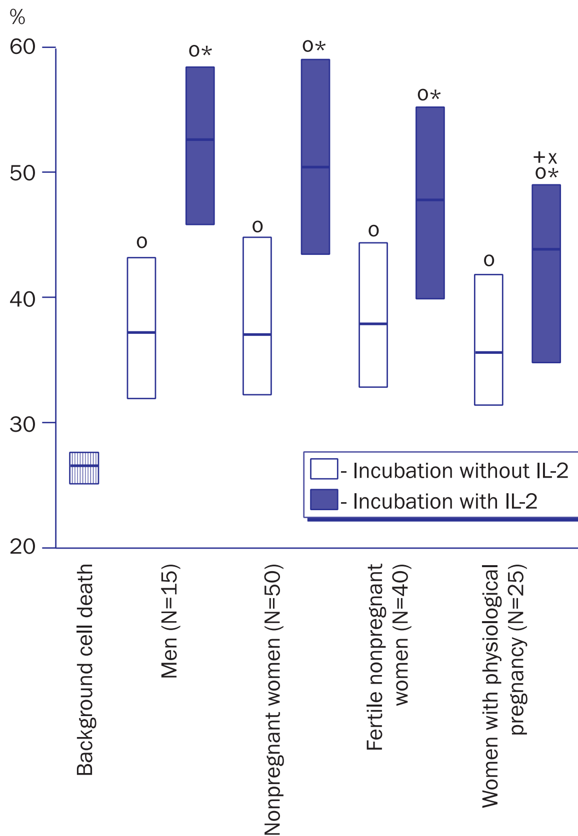

Fig. 3. Cytotoxic activity of NK cells in peripheral blood mononuclear cell fraction towards trophoblast cells. °*p*<0.001 in comparison with background trophoblast cell death; **p*<0.001 in comparison with incubation without IL-2; ˣ*p*<0.001 in comparison with cells from fertile nonpregnant women under similar culturing conditions; ⁺*p*<0.001 in comparison with cells from nonpregnant women under similar culturing conditions.

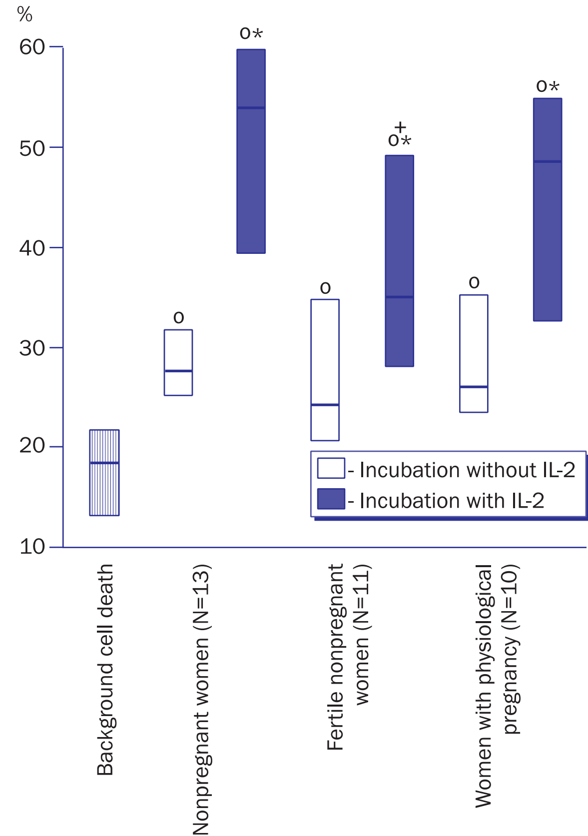

Fig. 4. Cytotoxic activity of NK cells isolated from peripheral blood mononuclear cell fraction towards trophoblast cells. °*p*<0.001 in comparison with background trophoblast cell death; **p*<0.001 in comparison with incubation without IL-2; ⁺*p*<0.001 in comparison with cells from nonpregnant women without previous pregnancies under similar culturing conditions.

(T-bet, Eomes, and Helios [10,20]) and intracellular messengers (ERK, JNK [20]), which restricts excessive cytotoxic function of NK cells.

According to published data, IL-2 enhances cytotoxic activity of NK cells [14]. IL-2 and IL-15 cytokine receptors consist of three subunits; two of these are $\beta\gamma_c$ chains homologous for both receptors [9]. Common subunits of IL-15 and IL-2 receptors, as well as common signal pathways activated by these cytokines [13] allow using them as partially interchangeable. It seems likely that the combination of exogenous IL-2 and trophoblast cells capable of IL-15 secretion not only activates NK cells, but also modulates their functional state. We found that in women with physiological pregnancy, the cytotoxic effect of NK cells in mononuclear cell fraction preincubated with IL-2 on trophoblast cells was less pronounced than in healthy nonpregnant women without previous pregnancies and healthy fertile women under similar culture conditions (Fig. 3). According to published reports [27], NK cells express receptors ILT and KIR2DL4 that bind HLA-G locus molecules expressed by trophoblast cells, thereby inhibiting activation of the cytotoxic function of NK cells. The established differences in the cytotoxic activity of NK cells in the presence of IL-2 in nonpregnant and pregnant women are probably the result of successful implantation during physiological pregnancy, after which the cytotoxic activity of NK cells is suppressed and their regulatory function comes to the foreground [8].

Isolated NK cells after incubation with IL-2 exhibited similar cytotoxic activity in groups of women with physiological pregnancy and healthy nonpregnant women (Fig. 4). In the group of fertile women, cytotoxic activity of isolated NK cells was lower than that in the group of nonpregnant women and was close to that in women with physiological pregnancy (Fig. 4). These results attest to changes in the functional status of NK cells in women who were previously pregnant. This difference can be determined by contacts of NK cells and trophoblast cells during previous pregnancies. Possible existence of memory-like NK cells that

can be formed both by CD56^{bright} NK cells and CD-56^{dim} NK cells is now discussed [23]. Among the possible variants of the formation of memory-like NK cells, special emphasis is laid on differentiation as a result of induction by cytokines [23]. During previous pregnancies, CD56^{bright} NK cells of the decidua could form memory-like NK cells under the influence of cytokines. The interaction of NK cells with trophoblast cells during previous pregnancies in fertile women can modify the behavior of NK cells in the presence of IL-2 activator.

Our findings on the cytotoxic activity of NK cells towards trophoblast cells both isolated and in mononuclear fraction, attest to additional modulation of the function of NK cells by other mononuclear cells. In case of mononuclear fraction, the cytotoxic activity of NK cells in women with physiological pregnancy was lower than in nonpregnant women and fertile women, which was not observed for isolated NK cells. The additional inhibitory effects are probably caused by cytokines secreted by other mononuclear cells. It was shown that macrophages secrete TGFβ [7] maintaining the pool of T-regulatory lymphocytes and involved in immunoregulatory function [17]. TGFβ is also secreted directly by T-regulatory cells [21], which seems to enhance the inhibitory effect of trophoblast cells on the cytotoxic function of NK cells in mononuclear fraction. In case of isolated NK cells, the absence of additional inhibitory signals from T lymphocytes and macrophages can explain the absence of difference in the cytotoxic activity of isolated NK from fertile women and pregnant women. Both T lymphocytes and macrophages are present in the decidua during pregnancy [3,11], which approximates our model system to the *in vivo* conditions.

These results drove us to a conclusion on a relationship between the functional status of peripheral blood NK cells and pregnancy. Reduced cytotoxic activity of NK cells towards trophoblast during pregnancy can underlie successful implantation, invasion of the blastocyst, and placenta formation. The modulation of the cytotoxic function of NK cells during pregnancy can be determined either directly by their interaction with trophoblast cells or by the influence of other mononuclear cells present in the decidua.

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