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Influence of tumor cells on natural killer cell phenotype and cytotoxicity

Natural Killer (NK) cells are known to lyse tumor cells lacking MHC-I and expressing ligands for activating receptors, thereby participating in cancer immune surveillance. However, their function and phenotype change significantly in cancer patients. The precise molecular mechanism(s) involved in this phenomenon remain unknown. The aim of the present study was to investigate changes of both receptor phenotype and cytotoxic activity of NK cells co-cultured with K562 and HepG2 cells in dual-chamber Transwell® plates. A decrease of NK cell cytotoxicity against K562 cells was demonstrated. This effect was not a result of changes in perforin, granzyme or CD107a expression by NK cells or by production of cytokines (IFNγ, TNFα, IL-10, and TGFβ). No changes in the expression of a majority of NK cell receptors (CD16, CD69, 2B4, NKG2A, NKG2D, NKp30, NKp44, NKp46, and DNAM1) were observed. Only an increase in the percentage of NK cells bearing inhibitory receptor TIGIT was found as a result of co-culture with tumor cells. Possible molecular mechanisms of altered NK cell cytotoxicity involving TIGIT are discussed.
Key words: NK cells, cytotoxicity, cancer immune surveillance.

Introduction

Natural Killer (NK) cells play a key role in immune surveillance in cancer. They dispatch susceptible cells by various mechanisms, including antibody-dependent cellular cytotoxicity, receptor-mediated induction of apoptosis, and triggered release of cytotoxic granules containing perforin and granzyme [1]. Initiation of killer NK cells begins with recognition of specific ligands expressed on target cells by a panel of receptors functionally categorized as activating or inhibitory. The NK cell response represents a net result of the interplay of signals from these activating and inhibitory receptors [2].

NK cell function and phenotype are severely altered in various types of malignant tumors, such as colorectal, breast, lung, hepatic, and others [3-6]. Various soluble factors secreted by tumor cells and their immunosuppressive microenvironment, including Treg cells, myeloid-derived suppressor cells, tolerogenic dendritic cells, and others, contribute to these changes [3]. However, NK cell function and phenotype change significantly in cancer

patients, and the molecular mechanism(s) involved remain unknown.

The aim of the present study was to study changes of both receptor phenotype and cytotoxic activity of human NK cells co-cultured with K562 and HepG2 cells in dual-chamber Transwell® plates. Results revealed a decrease of NK cell cytotoxicity under the influence of soluble tumor factors, accompanied by an increase in the percentage of NK cells expressing inhibitory receptor TIGIT.

Materials and methods

Blood samples and cell lines

This study was approved by relevant institutional review boards and all volunteers gave written informed consent according to the Helsinki Declaration. Peripheral blood samples were obtained from healthy volunteers. Human erythroleukemia K562 and hepatocarcinoma HepG2 cell lines were purchased from ATCC (USA). Cells were cultured in complete culture medium consisting of RPMI-1640 supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml

streptomycin (Sigma-Aldrich, USA) at 37°C and 5% CO_2. Before use, adherent HepG2 cells were detached from Petri dishes with a 0.05% trypsin solution and washed in RPMI-1640 medium.

Antibodies

The following labeled anti-human monoclonal antibodies (mAbs) were used for flow cytometry: anti-CD3-FITC, anti-CD56-PE, anti-CD56-PerCP, anti-CD107a-PerCP-Cy5.5, and anti-CD16-PE (Biolegend, USA); anti-NKp44-APC, anti-TIGIT-biotin, anti-DNAM-1-APC, anti-NKp46-APC, anti-CD69-FITC, anti-Perforin-APC, anti-NKp30-APC, anti-2B4-FITC, and anti-NKG2D-APC (Miltenyi Biotec, Germany); anti-NKG2D-PE and anti-Granzyme-PE (BD Biosciences, USA); anti-NKG2A-PerCP (R&D Systems, USA). Streptavidin-PE (R&D Systems, USA) was used for detection of cells linked to anti-TIGIT-biotin.

Cell separation

Peripheral blood mononuclear cells were isolated from venous peripheral blood by Hystopaque-1077 (Sigma-Aldrich) density gradient centrifugation. NK cells were purified by immunomagnetic separation using an NK Isolation Kit and VarioMACS cell separator (Miltenyi Biotech) according to the manufacturer's instruction. The purity of $CD3^-CD56^+$ cell populations exceeded 90% as estimated by flow cytometry.

Design of experiment in Transwell plates

NK cells (5×10^5) were placed in the bottom chamber of dual-chamber Transwell® plates (Sigma-Aldrich) in 1750 µl of complete culture media. Upper wells were filled with 250 µl of culture media containing 5×10^5 HepG2 or K562 cells. Only NK cells were incubated in control wells. Co-culture lasted for 48 h at 37 °C and 5% CO_2, then NK cells were collected, labeled with mAbs, and subjected to flow cytometry. The following receptors were analyzed on gated $CD3^-CD56^+$ cells: CD16, NKG2A, NKG2D, NKp30, NKp44, NKp46, CD69, DNAM1, TIGIT, and 2B4.

Flow cytometry

For surface and intracellular staining, cells were first incubated with mAbs specific for surface markers according to the manufacturer's protocols, then fixed and permeabilized with Fixation/Permeabilization solution (BD Pharmingen, USA), mixed well, and incubated for 20 min in the dark at room temperature. Cells were then washed with Perm/Wash Buffer (BD Biosciences) and stained with mAbs specific for intracellular molecules. Afterwards, cells were washed with phosphate-buffered saline, resuspended in flow solution,

and immediately analyzed by flow cytometry with FACSCalibur (BD Biosciences) and CellQuest software (BD Biosciences).

NK cell cytotoxicity assay

A 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess NK cell cytotoxicity. After incubation with K562 cells, HepG2 cells, or alone in Transwell® plates, NK cells were collected, counted, and a part of them were subjected to cytotoxicity assay. K562 cells were used as a target. Effector and target cells in a 2:1 ratio were co-cultured in round bottom 96-well plates (Sigma-Aldrich) in 200 µl of complete culture medium for 72 h. Both NK and K562 cells alone were used as controls. Until the last 4 h of cultivation, 50 µl of MTT stock solution (500 µg/ml; Sigma-Aldrich) was added. After cultivation, supernatants were removed and formazan crystals were dissolved in 150 µl DMSO (Sigma-Aldrich) and absorbance determined at an optical density (OD) of 492/630 nm. The index of cytotoxicity (IC) was calculated as follows:

$$IC(\%) = 100 \left(\frac{[C_{NK} + C_{K562} - E]}{[C_{NK} + C_{K562}]} \right),$$

where C_{NK} and C_{K562} are the OD of control wells and E is the OD of the experimental well.

NK cell cytotoxicity against K562 cells was also assessed by surface staining for CD107a and intracellular staining for perforin and granzyme B. For this, NK cells were co-cultured together with K562 cells as described above and then stained with anti-CD107a antibodies and incubated for 1 h, after which Brefeldin A (Biolegend, USA) was added according to manufacturer's instruction. After 3 hours, cells were collected, washed with phosphate-buffered saline, and subjected to intracellular staining for perforin and granzyme B according to manufacturer's instruction.

Cytokine secretion

Supernatants from co-cultures in Transwell® plates were collected and frozen at -20 °C. Sandwich ELISA was performed using human IL-10, IFNγ (BD Biosciences), and TGFβ1 (R&D Systems) ELISA sets according the manufacturer's instructions.

Statistical analysis

Data are represented as mean values ± quadratic error. The Student's t-test was used to analyze data. P-values of 0.05 were chosen as the limit of statistical significance.

Results and their discussion

Analysis of NK cell cytotoxicity after co-culture with tumor cells

The cytotoxicity of NK cells against K562 cells is considered a gold standard for assessment of NK cell activity. In our study, the IC of NK cells after co-culture with HepG2 and K562 cells approached 52.2±9.3% and 53.4±10.3%, respectively, contrary to 65.5±10.8% in the absence of co-cultured tumor cell lines. Since donor NK cells naturally differ by their primary cytolytic activity, we normalized IC values to controls (NK cells incubated alone in Transwell® plates), which were considered as 100%. Thereafter, NK cell cytotoxicity after incubation with both tumor cell lines reached 20% (Fig. 1).

Perforin and granzyme, along with death receptor family ligands, are executioners of NK cell mediated cytotoxicity. CD107a is a marker of degranulation highly correlated with emptying or NK cell cytotoxic granules [7]. Unexpectedly, we did not find statistically significant differences in CD107a, perforin, or granzyme expression in NK cells co-cultured with tumor cell lines (Table 1).

Phenotyping of NK cells

Transwell® plates allow free exchange of soluble factors and secreted exosomes between co-cultured cells. We examined alterations of NK cell activating (DNAM-1, NKG2D, NKp30, NKp44, NKp46) and inhibitory receptors (TIGIT, NKG2A), as well as CD16 (FcR□III), involved in antibody-dependent cellular cytotoxicity. However, changes in the expression of a majority of receptors/markers did not reach statistical significance (Table 2).

Taking into account the complex interplay between TIGIT and its activating counterpart, DNAM-1 [8], and their role in tumor surveillance, we analyzed independent and co-expression of DNAM-1 and TIGIT. A significant increase of NK cells expressing TIGIT alone after co-culture with both HepG2 and K562 cells was observed (Table 3). Levels of DNAM-1$^+$TIGIT$^-$ and DNAM-1$^+$TIGIT$^+$ NK cells did not change significantly (Table 3, Fig. 2).
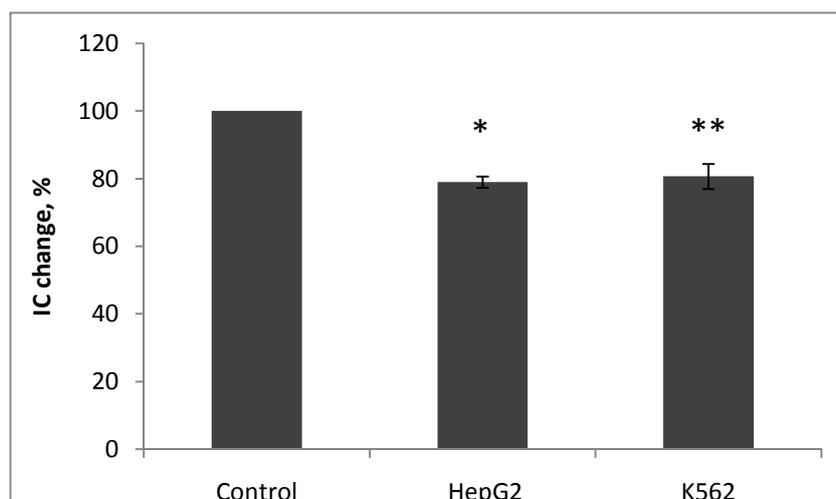
Figure 1 – Cytotoxicity of NK cells against K562 target after co-culture with HepG2 or K562
tumor cell line, assessed by MTT test. Data are normalized as percent of cytotoxicity index (IC) of NK cells,
cultured in Transwell® plates alone, and are represented as M±m, n=3.
*P=0.007, **P=0.038

Table 1 – Percent of NK cells expressing molecules, associated with cytotoxicity

Items	Variants of test	Granzyme	Perforin	CD107a
1	NK alone	85.9±2.7	93.4±1.0	99.2±0.4
2	NK + HepG2	80.0±7.2	89.2±2.8	94.4±5.3
3	NK + K562	80.5±3.3	89.6±1.8	96.1±3.0
4	n	3	4	5
5	P$_{1-2}$	P>0.05		
6	P$_{1-3}$	P>0.05		

Table 2 – Percent of NK cells expressing particular receptors

Items	Variants of test	CD16	CD69	2B4	NKG2A	NKG2D	NKp30	NKp44	NKp46
1	NK alone	69.9±11.5	2.5±0.4	86.5±21.7	4.1±2.3	76.3±5.6	5.7±2.4	1.8±0.8	89.5±3.2
2	NK+HepG2	79.8±1.3	5.3±2.4	81.2±10.2	3.5±1.8	76.6±8.7	7.4±3.4	2.9±1.3	91.0±3.6
3	NK+K562	73.4±6.0	8.5±5.4	82.8±10.5	7.1±3.0	69.9±11.6	3.5±0.9	2.7±0.6	89.1±4.4
4	n	5	7	7	5	6	7	6	5
5	P_{1-2}	P>0.05							
6	P_{1-3}	P>0.05							

Table 3 – Percent of NK cells, expressing DNAM-1, TIGIT or their combinations, by NK cells

Items	Variants of test	DNAM-1	TIGIT	DNAM-1$^+$TIGIT$^+$
1	NK alone	94.8±2.0	4.7±1.4	6.1±2.4
2	NK+HepG2	94.2±1.2	10.4±2.5	16.9±5.1
3	NK+K562	95.6±1.1	12.0±2.5	18.8±6.7
4	n	3	5	3
5	P_{1-2}	0.811	0.042	0.204
6	P_{1-3}	0.767	0.015	0.273

Analysis of cytokine production

Cytokines are known to modulate NK cell activity. We analyzed participation of proinflammatory (IFNγ, TNFα) and anti-inflammatory (IL-10, TGFβ) cytokines in modulation of NK cytotoxicity by determining their concentrations in no-culture supernatants. No changes in the concentration of any of these cytokines were found in the culture media after NK cell co-culture with tumor cells (Table 4).

The functional incapacitation of NK cells infiltrating tumors is well established [5]. Much research has been performed to analyze NK cell peculiarities in the presence of tumor cells *in vitro* and in cancer patients, but precise molecular mechanism(s) of this phenomenon remain unknown. Theoretically, two influences could alter NK cell cytolytic activity: cell-to-cell contact and/or the impact of soluble factors. In this study, we analyzed the influence of possible soluble factors secreted by two different tumor cell lines during co-culture with NK cells in dual-chamber Transwells® on NK cytolytic activity, as well as NK cell receptor/marker expression and cytokine production.

Table 4 – Cytokine levels in conditioned media, pg/ml, M±m.

Items	Variants of test	IFNγ	TNFα	IL-10
1	NK alone	1579±43	170±3	257±8
2	NK+HepG2	1630±11	170±2	266±4
3	NK+K562	1707±40	182±6	268±14
4	n	3	3	3
5	P_{1-2}	P>0.05		
6	P_{1-3}			
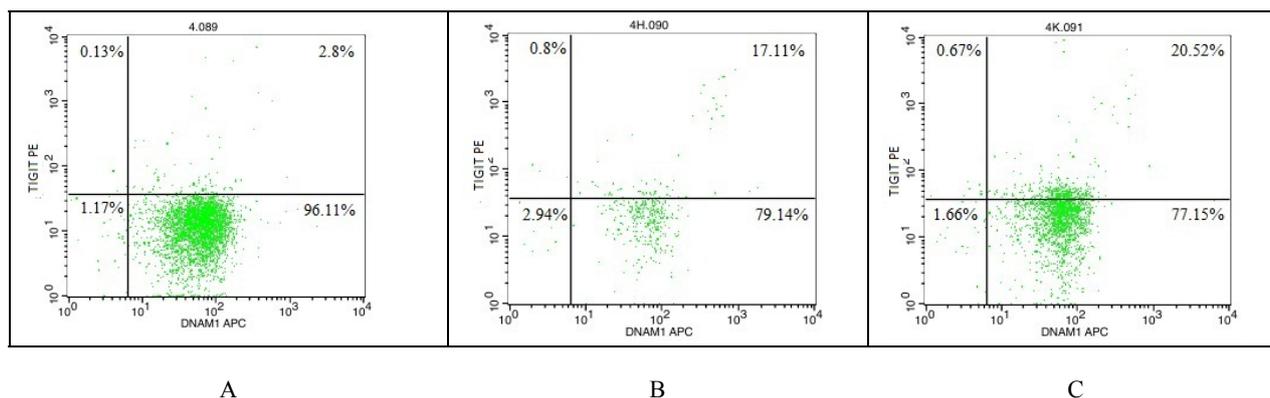
Figure 2 – Representative data of flow cytometry showing co-expression of DNAM-1 and
TIGIT on NK cells after Transwell® co-culture experiments. A – NK cells cultured alone;
B – NK cells after co-culture together with HepG2 cells; NK cells after co-culture together with K562 cells

A decrease in NK cell cytotoxicity to K562 cells after co-culture with either HepG2 or K562 cells probably involved certain soluble tumor factors. We showed that the decrease of NK cell activity was not associated with an alteration in perforin and granzyme expression, two proteins known to mediate the cytolytic impact of NK cells. Moreover, we did not observe changes in CD107a presentation on the NK cell surface. These results indicate the fundamental readiness of NK cells to cytolytic activity.

Taken together, decreased NK cell function possibly results from deterioration of target ligand recognition by NK cell receptors due to inhibition of receptor expression. Our analysis of a panel of NK cell receptors (CD16, CD69, 2B4, NKG2D, NKp30, NKp44, NKp46) did not elucidate any changes in their expression after co-culture with tumor cell lines. In addition, there was no change in the expression of inhibitory receptor NKG2A. However, we did detect an increase in the expression of another inhibitory receptor, TIGIT, on NK cells after co-culture with HepG2 and K562 cells. This finding is of particular interest because the NK cell response is determined by the net outcome of signals from activating and inhibitory receptors [2]. Many NK cell activating or inhibitory receptors share a common ligand [8, 9]. Distinctive examples are DNAM-1, TIGIT, and CD96, all critical regulators of NK cell function which share CD155 and CD112 ligands (members of nectin and nectin-like protein families) [10]. CD155 is a dominant ligand over CD112 for DNAM-1 and TIGIT [11]. CD155 (Necl5, PVR, Tage4) is an immunoglobulin-like receptor found on certain tumors [10, 12, 13].

Although functional involvement of TIGIT in altered NK cytotoxicity was not examined, our findings could theoretically be explained as follows. HepG2 and K562 cells express CD112 and CD155 [13] and secrete exosomes [14]. If CD112 or CD155 are enriched on exosomes or secreted as free soluble forms and bind TIGIT, thereby activating this inhibitory receptor, its engagement could depress NK cell cytotoxicity. However, this hypothesis does not explain the observed increase in the percentage of TIGIT+ NK cells.

Recently it has been shown that K562 cells can secrete exosomes rich in microRNAs, many of which are involved in modulation of Wnt signaling pathways [14], which could change transcription of the gene encoding TIGIT. The role of Wnt signaling has been investigated previously in differentiation of NK cell lineages [15]. It is of interest to study other facets of this pathway in NK cell biology.

In conclusion, our study demonstrates an increase of NK cells expressing inhibitory receptor TIGIT that may decrease the natural cytotoxicity of these cells. It is of interest to determine whether this phenomenon is recapitulated in cancer patients and could represent a novel parameter in assessment of NK cell function.

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