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CD56^{dim}/CD56^{bright} NK Cell Subpopulations and CD16/CD57 Expression Correlated with Tumor Development Stages

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Abstract

BACKGROUND: NK cells are characterized by cytotoxic activity against tumor cells and CD3-CD16+CD56+ phenotype. Two distinct subpopulations of NK cells were characterized in the peripheral blood: NK-CD56^{dim} representing over 95% of NK cells and involved in antitumor cytotoxicity, and NK-CD56^{bright} representing approximately 10% of NK cells and involved in secretion of cytokines.

AIM: The aim of the study was to compare the presence of NK-CD56^{dim}/NK-CD56^{bright} subpopulations and their CD16/CD57 expression in peripheral blood NK cells during the particular development stages of malignancy: primary tumor (PT), lymph node invasion (LNI) and distant sites metastases (Mt).

MATERIAL AND METHODS: We have analyzed by flow-cytometry peripheral blood samples from total 36 cancer patients: 24 patients with PT, 6 patients with LNI and 6 patients with Mt.

RESULTS: The presence of the overall NK cells showed no significant variation between patients in different stages of tumor development. The phenotype analysis showed that CD16+ and/or CD57+ cells were lower in LNI patients compared to PT or Mt patients. Double-positive CD16+CD57+ cells were found decreased in patients with Mt, compared to patients with PT. During the stages of tumor development, NK-CD56^{bright} subpopulation increased progressively (7% in PT patients, 13% in LNI patients, 65% in Mt patients), whereas NK-CD56^{dim} subpopulation gradually decreased (92%, 86%, and 35% respectively). CD16/CD57 expression decreased in NK-CD56^{dim} and increased in NK-CD56^{bright} cells over the three studied stages.

CONCLUSION: Our results show changes in NK cells characteristics during tumor development: reversal of NK-CD56^{dim}/NK-CD56^{bright} distribution and modification of CD16/CD57 expression. Both types of changes can concur in reducing the efficiency of NK cell activity in patients with progressive tumors.

Introduction

NK cells are characterized as large granular lymphocytes with the CD3^{-}CD16^{+}CD56^{+} phenotype [1-3] and antitumor cytotoxic activity [4-7]. Other molecules associated with these cells have been described, such as adhesion molecule CD57, cytotoxicity receptors KIR (CD158), LIR (CD85), NKG2 (CD94, CD315) and NKP (CD335, CD336) [8-11]. During the last decade, two distinct subpopulations of NK cells were characterized as having primary phenotypic expression associated with a specific function: NK-CD56^{dim} (CD56$^{dim/neg}$, CD56^{low}) and NK-CD56^{bright} (CD56^{high}) [12-14]. NK-CD56^{dim} cells comprise the majority of peripheral blood and spleen NK cells (> 95%), they have CD16$^{+/-}$CD57^{-} KIR^{+}NKP^{+} phenotype and are involved in antitumor cytotoxicity [15, 16]. NK-CD56^{bright} cells represent approximately 10% of all NK cells, are mainly located in the lymph nodes and tonsils, express predominantly CD16$^{+/-}$CD57^{-}KIR^{-} phenotype, lacking cytotoxicity receptors but are involved in the active secretion of cytokines [17-19].

The particular distribution of NK subpopulations can be significant and suggestive

when evaluating their cytotoxic potency as vigorous antitumor cellular immune response players. Some deficiencies in the function of tumor-infiltrating NK cells have been observed [20-22], showing that the tumor microenvironment may play an important role in the development of NK cytotoxicity. However, a relationship between the intratumoral NK cell functions and NK-CD56^{dim}/ NK-CD56^{bright} subsets could not be clearly established. In contrast, significant features were recently discovered by investigating the distribution of NK blood subsets. The reported results indicate that predominance of peripheral CD56^{dim}CD16^{+}KIR^{+} NK cells constitutes an end result of the recruitment of NK-CD56^{bright}CD16^{-} KIR^{-} in the local immune response [23]. This phenomenon seems to be facilitated by chemokines [24] and upon IL-2, IL-12 or IL-15 stimulation [25]. Although some particular aspects of NK subpopulations distribution in the peripheral blood have been already observed in viral diseases [26, 27], pregnancy [28, 29] and even in autoimmune diseases [30], data regarding their value in the diagnosis of cancer are scarce.

Based on our preliminary studies [31], we now attempt to compare different aspects of the phenotype and peripheral NK cell subpopulations along the three progressive stages of tumor development: primary tumor (PT), lymph node invasion (LNI), metastases (Mt). We consider that, among other features, the diagnosis and monitoring of tumor development depend on the assessment of changes in the distribution of NK cells.

Materials and Methods

Patients

Whole peripheral blood samples were collected from 36 cancer patients (24 patients with PT, 6 patients with LNI and 6 patients with Mt). All subjects gave informed consent before their inclusion in the study.

PTs were 10 retroperitoneal, 2 kidney, 2 bladder, 4 colons, 2 gastric, 2 breasts, 1 ovarian, 1 pelvis. There were 11 man and 13 women, average age 62. The patients with LNI had either osteosarcoma, breast, squama cellular, colon, renal and perineal tumor. There were 4 man and 2 women, average age 63. Mt came from 3 colorectal, 1 gastric, 1 breast and 1 retroperitoneal carcinoma. There were 4 man and 2 women, average age 65.

No patient supported immunomodulatory treatment at the moment of testing. Blood samples from 10 healthy donors served as controls.

The study was approved by the institution ethics committee.

Lymphocyte immunophenotyping

All patients and control subjects were tested for lymphocyte populations (T, B and NK cells) by flow cytometry immunophenotyping at FACSCalibur cytometer (BD Biosciences, San Jose, CA, USA), using 4-colour automatic methodology. The blood was processed with MultiTest IMK Kit (BD Biosciences) according to manufacturer's instructions. Data acquisition was performed using BD MultiSet software.

Characterization of NK cells

Given that MultiTest immunophenotyping kit evaluates only CD16^{+} NK cells subset, we performed an additional analysis of NK cell phenotype by assessing CD16/CD57 expression and CD56^{dim}/CD56^{bright} subpopulations. The analysis was performed in 8 patients with PT and all patients with LNI and Mt. The peripheral blood samples were labeled with CD3PerCP, CD56APC, CD16PE and CD57FITC (BD Biosciences) and then phenotyped at FACSCalibur cytometer by the 4-color manual method, using BD CellQuest software for data acquisition (Figure 1). All monoclonal antibodies were used according to manufacturer's instructions as previously described [32].

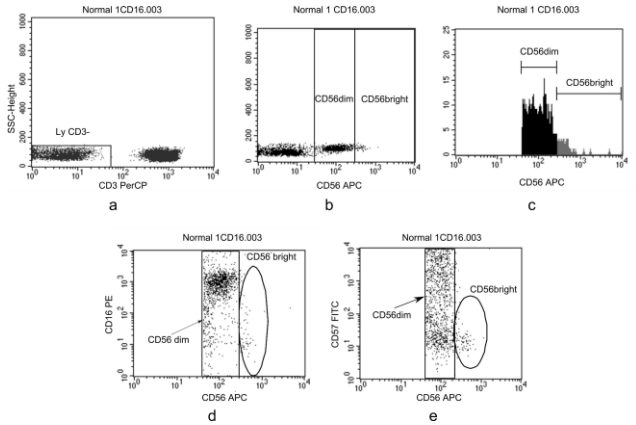

Figure 1: Flow cytometric dot plot analysis of NK cell subsets.The determination with BD FACSCalibur cytometer by manual 4-color method, data acquisition with BD CellQuest software. a: CD3^{-} cells of total lymphocytes, b: CD3^{-}CD56^{+} cells (NK cells), c: NK-CD56^{dim} and NK-CD56^{bright} subpopulations in CD3^{-}CD56^{+} cells, d: CD16^{+} phenotype in NK cell subpopulations, e: CD57^{+} phenotype in the NK cell subpopulations

Statistical analysis

The results are presented as the mean ± standard deviation of cell percentages.

The T, B and NK cells were measured as a percentage of total lymphocytes. Normal ranges are 5th to 95th percentile, as stated in Table 1.

NK subpopulations and cell phenotypes were calculated as percentages of total CD56^{+} cells.

Graphs and t-test were performed using Microsoft Excel software.

Results

Lymphocyte immunophenotyping

The populations of peripheral blood lymphocytes revealed some differences in the distribution of cells between the three stages of tumor development (Table I). Thus, CD4$^+$ T-cells and B cells were higher in patients with PTs compared to the level observed in patients with Mt. CD8$^+$ T cells were higher in patients with Mt, compared to the patients with PT. In addition, T-CD4+: T-CD8+ ratio was lower in LNI and Mt patients than in patients with PTs.

Table 1: Lymphocyte percentages in peripheral blood of tested subjects

Cells	Controls	PT patients	LNI patients	Mt patients	Normal range
T-CD3$^+$	69 ± 6	67 ± 13	78 ± 11	70 ± 12	56 – 86
T-CD4$^+$	41 ± 5	40 ± 11	40 ± 9	34 ± 6****	33 – 57
T-CD8$^+$	28 ± 7	26 ± 14	36 ± 9	36 ± 12****	13 – 39
T-CD4+:T-CD8+ ratio	1.6 ± 0.4	2.0 ± 1.0	1.2 ± 0.5***	1.0 ± 0.3*	1.5 – 2.5
B	13 ± 3	12 ± 5	6 ± 3**	9 ± 3***	6 – 22
NK	16 ± 5	19 ± 14	16 ± 13	21 ± 11	5 – 25

[a] 5th to 95th percentile, as presented by MultiTest IMK Kit manufacturer; * p < 0.001 compared to PT patients; ** p < 0.01 compared to PT patients; *** p < 0.02 compared to PT patients; **** p < 0.05 compared to PT patients.

The distribution of NK cells did not show any significant variation between control subjects and patients with PTs. Also, no significant imbalance has been observed during the stages of tumor development in terms of percentage of NK cells.

Characterization of NK cells

a. CD16/CD57 expression

Total NK cells (CD56$^+$) were further examined to determine CD16$^+$ or CD57$^+$ phenotype (single-positive cells) and CD16$^+$CD57$^+$ phenotype (double-positive cells). The results are shown in Table 2.

Irrespective of CD16$^+$ or CD57$^+$ phenotype, the percentages of single- or double-positive cells were the lowest in patients with LNI as compared to controls or PT and Mt patients. As considering the three tumor development stages, the percentages of single- or double-positive cells were significantly higher in patients with PT, with no significant difference when comparing to controls.

Table 2: NK cell phenotypes during tumor development stages

	NK cell phenotypes (% cells)			
	CD56$^+$ [a]	CD56$^+$CD16$^+$ [b]	CD56$^+$CD57$^+$ [b]	CD56$^+$CD57$^+$CD16$^+$ [b]
Controls	15 ± 6	93 ± 5	53 ± 13	52 ± 12
PT patients	16 ± 12	86 ± 11	71 ± 17	65 ± 19
LNI patients	10 ± 4	39 ± 28*	36 ± 18**	20 ± 18*
Mt patients	26 ± 10	51 ± 17**	64 ± 12***	49 ± 18

[a] Percentage in all lymphocytes; [b] Percentage in CD56$^+$ cells; * p < 0.001 compared to PT patients and controls; ** p < 0.01 compared to PT patients; *** p < 0.05 compared to PT patients.

b. CD56^{dim}/CD56^{bright} subpopulations

During the stages of tumor development, the distribution of NK-CD56^{dim}/ NK-CD56^{bright} subpopulations changed gradually (Figure 2). The patients with PT showed a similar distribution with the control subjects, i.e. NK-CD56^{dim} was the predominant subpopulation (93%). The percentage of NK-CD56^{bright} subpopulation gradually increased from 7% in patients with PT to 13% in patients with LNI and 65% in patients with Mt (p <0.001), while NK-CD56^{dim} subpopulation was decreasing.

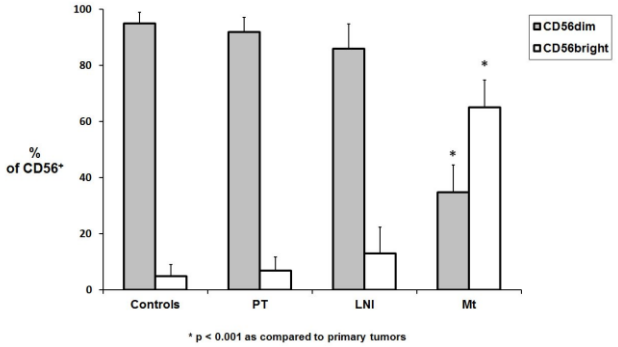

Figure 2: Changes in the distribution of NK-CD56^{dim}/ NK-CD56^{bright} subpopulations according to the period of tumor development

NK-CD56^{dim}/ NK-CD56^{bright} subpopulations showed similar variation as above in terms of CD16/CD57 phenotypes (Figure 3). In NK-CD56^{dim} subpopulation, CD16$^+$/CD57$^+$ expression progressively decreased as follows: CD16$^+$ cells from 89 ± 12% in PT patients to 11 ± 9% in Mt patients; CD57$^+$ cells from 73 ± 16 % in PT patients to 10 ± 6% in Mt patients; CD16$^+$CD57$^+$ cells from 68 ± 19% in PT patients to 7 ± 8% in Mt patients (Figure 3a).

In NK-CD56^{bright} subpopulation, the CD16$^+$/CD57$^+$ expression progressively increased as follows: CD16$^+$ cells from 40 ± 33% in PT patients to 91 ± 8% in Mt patients; CD57$^+$ cells from 28 ± 30% in PT patients to 71 ± 20% in Mt patients; CD16$^+$CD57$^+$ cells from 28 ± 30% in PT patients to 70 ± 20% in Mt patients (Figure 3b).

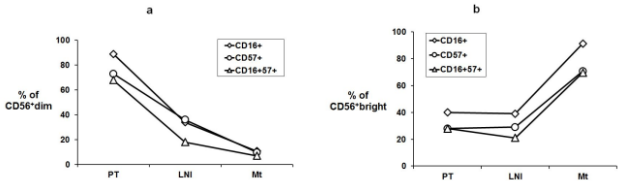

Figure 3: CD16/CD57 phenotypes in NK subpopulations during tumor stages. a: Changes in the phenotype of NK-CD56^{dim} subpopulation, b: Changes in the phenotype of an NK-CD56^{bright} subpopulation

It must be emphasized that the values are not statistically significant because the three groups did not include the same patients. However, they could highlight a possible trend in expression of CD16/CD57 markers along the evolutionary phases of the tumor.

Discussion

We have focused on assessing the NK cell population in terms of its representation during the progressive stages of malignancy, from primary tumors, lymph nodes involvement to distant sites metastasis (PT, LNI, Mt). It is known that NK cells are actively involved in the anti-tumoral response, hence, we wanted to evaluate NK cell phenotype and their subpopulations distribution in the peripheral blood as possible indicators of tumor development to more advanced stages. At the same time, evaluating the specific phenotype in relation to tumor evolution, new markers indicating antitumor cellular cytotoxicity could be revealed. We have purposely chosen different solid cancers to highlight that stages of tumor development can have common immune-related mechanisms, possibly sustained by a clear anti-tumoral cell like NK population.

The above-mentioned stages of tumor progression we considered were mainly clinical, concerning that they were diagnosed by standard clinical/paraclinical methods. Therefore, an auxiliary method as the determination of NK cells supplies additional information, indicating how the tumor growth and evolution affect these cells. This could be important since the non-surgical anti-tumor therapy may target for specific NK activity improvement [22].

The percentages of peripheral blood NK cells varied slightly in patients at different stages of tumor development. In spite of this minor variation, other data can be provided by analyzing the expression of CD16 and CD57 molecules or distribution of NK-$CD56^{dim}$/ NK-$CD56^{bright}$ subsets. Although functional differences between the two NK subpopulations are well known [15, 17], relatively little evidence was provided about their participation in specific antitumor response and their possible assessment during tumor development.

We also have observed two changes in NK subpopulations during tumor evolution: a downward trend in the share of NK-$CD56^{dim}$ subpopulation and on the other hand a progressive increase of NK-$CD56^{bright}$ subpopulation. Taking into account that NK-$CD56^{dim}$ cells exert largely cytotoxic actions, we assume that tumor progression could be favored by the decrease of this subpopulation. Given that the increased NK-$CD56^{bright}$ cells develop regulatory activities, it can be concluded that they are not very effective in the recruitment of other active cytotoxic cells, such as $CD8^+$ T cells, which are essential to the eradication of metastatic tumors [33]. Even if we found significantly increased peripheral blood $CD8^+$ T cells in patients with Mt compared to PT patients, it is doubtful whether these cytotoxic cells are capable of working effectively. For this reason, we tend to assume that changing the NK-$CD56^{dim}$/NK-$CD56^{bright}$ balance in favor of NK-$CD56^{bright}$ cells may represent probably a warning sign for tumor escape.

Examining the CD16/CD57 phenotype, we observed that the percentages of positive NK cells were significantly lower in patients with LNI compared to the levels observed in PT or Mt patients. Knowing that both CD16 and CD57 molecules are involved in intercellular interactions, we can emphasize that this decrease could be an issue in NK cell adhesion during tumor growth, especially in the lymph node invasion process, which seems to be a critical moment. This phenomenon may be exacerbated by the lower NK-$CD56^{dim}$ subpopulation with decreased CD16/CD57 expression and cannot be corrected by the increase of NK-$CD56^{bright}$ subset. Thus, such a hypothetical replacement of a cytotoxic subpopulation (NK-$CD56^{dim}$) with a regulatory subpopulation (NK-$CD56^{bright}$) cannot be beneficial in terms of cytotoxicity efficiency and anti-tumoral effect [5].

Our presented results pinpoint an ineffective change of the peripheral blood NK cells during tumorigenesis and tumor dynamics: gradual decrease of the NK-$CD56^{dim}$ and increase of the NK-$CD56^{bright}$ subpopulations. This could favor tumor invasion in the lymph nodes, ending with a reversal of the NK-$CD56^{dim}$/ NK-$CD56^{bright}$ distribution in the course of metastasis process. Changing in CD16/CD57 co-expression on NK cells can cause difficulties in efficient cytotoxicity by reducing the intercellular contact, a critical step in developing an anti-tumoral effect. Each of these changes contributes to the ineffectiveness of NK cells and hence tumor progression. These features can be sensed indirectly by phenotypic characterization of NK cells and their subpopulations and could be cellular markers of poor clinical outcome in solid tumors.

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