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Primary Human Tumor Cells Expressing CD155 Impair Tumor Targeting by Down-Regulating DNAM-1 on NK Cells

Mattias Carlsten,2✉ Håkan Norell,† Yenan T. Bryceson,* Isabel Poschke, † Kjell Schedvins,§ Hans-Gustaf Ljunggren,✉ Rolf Kiessling, † and Karl-Johan Malmberg2✉

The activating NK cell receptor DNAX accessory molecule-1 (DNAM-1) contributes to tumor immune surveillance and plays a crucial role in NK cell-mediated recognition of several types of human tumors, including ovarian carcinoma. Here, we have analyzed the receptor repertoire and functional integrity of NK cells in peritoneal effusions from patients with ovarian carcinoma. Relative to autologous peripheral blood NK cells, tumor-associated NK cells expressed reduced levels of the DNAM-1, 2B4, and CD16 receptors and were hyporesponsive to HLA class I-deficient K562 cells and to coactivation via DNAM-1 and 2B4. Moreover, tumor-associated NK cells were also refractory to CD16 receptor stimulation, resulting in diminished Ab-dependent cellular cytotoxicity against autologous tumor cells. Coincubation of NK cells with ovarian carcinoma cells expressing the DNAM-1 ligand CD155 led to reduction of DNAM-1 expression. Therefore, NK cell-mediated rejection of ovarian carcinoma may be limited by perturbed DNAM-1 expression on tumor-associated NK cells induced by chronic ligand exposure. Thus, these data support the notion that tumor-induced alterations of activating NK cell receptor expression may hamper immune surveillance and promote tumor progression.

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Natural killer cells are lymphocytes of the innate immune system that recognize and kill tumor cells without prior sensitization (1). Human NK cells can be divided into two main functional subsets based on the intensity of CD56 expression (2). Although there is a degree of functional overlap, CD56dim NK cells have a potent cytotoxic function, whereas perforinlowCD56bright NK cells are thought to have immunoregulatory properties (2–5). Recent data support the notion that CD56bright and CD56dim NK cells represent different stages of maturation, with CD56dim NK cells being the more differentiated cell type (6).

NK cells are regulated by a balance of activating and inhibitory signals from cell surface receptors (7). The inhibitory signals are mediated mainly by HLA class I-binding receptors, including killer Ig-like receptors (KIRs),3 CD94/NKG2A, and leukocyte Ig-like receptor B1 (LILR-B1) (8). Activating signals are encoded by a wide array of receptors, including NKG2D, DNAX accessory molecule-1 (DNAM-1), natural cytotoxicity receptors (NKP30, NKP44, NKP46), CD94/NKG2C, and KIR with activating intracellular domains (7). Additionally, the low-affinity FcγRIIIA receptor CD16 mediates Ab-dependent cellular cytotoxicity (ADCC). Costimulatory receptors and adhesion molecules such as 2B4 and LFA-1 are also involved in the regulation of NK cell activity (7). Engagement of specific combinations of activating receptors on NK cells dictates qualitatively distinct responses and can lead to synergistic effects for activation of effector cell function (9, 10).

The ability of NK cells to kill tumors in vitro has been taken as indirect evidence for their participation in tumor immune surveillance. However, more direct evidence for a role of NK cells in tumor immune surveillance is limited. In murine models, however, two groups have independently reported an increased risk of tumor development in mice lacking either the NKG2D or DNAM-1 receptor (11, 12). DNAM-1-deficient mice developed significantly more DNAM-1 ligand-expressing fibrosarcoma and papilloma tumors compared with wild-type mice in response to the chemical carcinogens methylcholanthrene and 7,12-dimethylbenz(a)anthracene (12). These results substantiate the notion of DNAM-1 playing an important role in immune surveillance of tumor development.

The DNAM-1 receptor is involved in the induction phase of both T and NK cell activation (13). Poliovirus receptor (CD155) and Nectin-2 (CD112) have been identified as ligands for DNAM-1, with CD155 appearing to have a predominant role in inducing DNAM-1-dependent responses (14). CD155 is widely expressed on normal cells and overexpressed on many tumor types (14–21). We recently demonstrated that the DNAM-1/CD155 interaction is crucial for recognition and killing of freshly isolated human ovarian carcinoma cells by resting allogeneic NK cells (15). Since the ovarian carcinoma cells constitutively express CD155 in combination with reduced levels of HLA class I molecules (15, 22, 23), autologous NK cells could theoretically target...
this tumor type. However, early studies suggest that the function of NK cells in patients with ovarian carcinoma is suppressed by an as yet unclear mechanism (24, 25).

Here, we have examined the activating receptor repertoire and function of tumor-associated NK (TANK) cells isolated from the tumor environment in patients with ovarian carcinoma. Our data reveal substantial alterations of the NK cell receptor repertoire, including significantly reduced expression of DNAM-1, 2B4, and CD16, resulting in impaired activation of NK cells and poor tumor cell targeting. Furthermore, we show that interactions with CD155-expressing target cells lead to reduction of DNAM-1 expression on NK cells. Taken together, our results provide a molecular mechanism that may contribute to impaired NK cell-mediated tumor rejection in ovarian carcinoma.

Materials and Methods

Cells

This study was approved by the institutional ethics committee (Karolinska Institutet, approval nos. 03-537 and 2007/1037-31/2). Patients with ovarian carcinoma anti-subjects to primary surgery before chemotherapy were included in this study. Patient characteristics are described in supplemental Table 1. Peritoneal effusions and peripheral blood samples were obtained with informed consent. Blood from healthy donors was obtained from the Blood Centre at the Karolinska University Hospital. Lymphocytes were enriched by density gradient centrifugation (Ficoll-Hypaque; Amersham Biosciences) as previously described (26). Cells were frozen in 10% DMSO (Sigma-Aldrich) and 90% heat-inactivated FBS (Invitrogen) and stored in liquid nitrogen. NK cells used in functional experiments were isolated from frozen PBMCs using the NK cell isolation kit II (Miltenyi Biosciences) and resuspended in complete medium (RPMI 1640 containing 10% heat-inactivated FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin) supplemented with 1000 IU/ml IL-2 (Proleukin; Chiron) and incubated overnight at 37°C before use. Fresh ovarian carcinoma cell lines were isolated from tumor samples and were positive magnetic bead selection with anti-PE kit (EasySep kit; StemCell Technologies) or isolated from peritoneal effusions using the CD45 depletion kit (StemCell Technologies). The human erythroleukemia cell line K562, the LCL line 721.221, the mouse mastocytoma cell line P815, and the ovarian adenocarcinoma cell lines SKOV-3 and CaOV-4 (American Type Culture Collection) were all maintained in complete medium. The Drosophila Schneider 2 (S2) insect cell line was maintained as previously described (27).

Abs and reagents

The following reagents and fluorescent-labeled mAbs were used. PMA and ionomycin were purchased from Sigma-Aldrich. GolgiPlug (brefeldin A), anti-NKG2D PE (1D11), anti-CD16 Alexa 647 (3G8), and anti-CD69 allophycocyanin (FN50), anti-CD56 PE-Cy7 (B159), anti-CD162 FITC and PE anti-NKG2D PE (1D11), anti-CD16 Alexa 647 (3G8), anti-CD226 FITC and PE anti-NKG2D PE (1D11), and anti-NKp46 (BAB281) (Immunotech). All other mouse anti-human Abs, except for anti-CD14 (MØP9; IgG2a) and anti-CD16 (3G8) (Beckman Coulter), anti-CD226 (DX11), anti-CD56 PE-Cy7 (B159), anti-CD107a mAbs for 15 min on ice, followed by washing and permeabilization (BD Biosciences). Intracellular staining was performed with anti-IFN-γ and anti-TNF-α mAbs before acquisition. P815 cells used in the reverse lysis assays were preincubated with agonistic Abs at a final concentration of 2.5 μg/ml. Abs used for ADCC were present in the assay at 10 μg/ml. Experiments involving redirected lysis or ADCC were run for 6 h. Experiments involving stimulation by PMA (200 μM) and ionomycin (1000 μg/ml) were stopped after 2 h of incubation.

Flow cytometry-based cytotoxicity assays

Fresh ovarian carcinoma cells were isolated and HLA class I was blocked by the anti-HLA class I mAbs A6-136 and 12B4 at the dilution 1/1 and 1/100, respectively (both mAbs were provided by Dr. D. Pende, Genoa, Italy). Purified overnight IL-2-activated NK cells were coincubated with target cells at a ratio of 10:1 in a final volume of 200 μl for 3 h at 37°C and 5% CO₂. At the end of the assay, tumor cells were stained with the anti-HLA class I mAbs A6-136 and 12B4 at the dilution 1/1 and 2B4 PE (C1.7), unconjugated anti-NKG2A (Z199), unconjugated anti-CD155 (PV.404.19), and IgG1 isotype control PE (679.1Mc7) were added together with primary labeled mAbs followed by incubation for 15 min on ice. Cells were washed twice and immediately analyzed on a CyAn ADP LX9 9 color flow cytometer (Dako). Isotype-matched control Abs were used at similar concentrations. The data were analyzed with FlowJo software (Tree Star).

Analysis of NK cell activation

NK cells were coincubated with target cells at a ratio of 1:1 in a final volume of 200 μl in 96-well plates at 37°C and 5% CO₂. After 1 h of coincubation, GolgiPlug was added. Before analysis, cells were stained with a dead cell marker and a combination of anti-CD3, anti-CD56, and anti-CD107a mAbs for 15 min on ice, followed by washing and permeabilization (BD Biosciences). Intracellular staining was performed with anti-IFN-γ and anti-TNF-α mAbs before acquisition. P815 cells used in the reverse lysis assays were preincubated with agonistic Abs at a final concentration of 2.5 μg/ml. Abs used for ADCC were present in the assay at 10 μg/ml. Experiments involving redirected lysis or ADCC were run for 6 h. Experiments involving stimulation by PMA (200 μM) and ionomycin (1000 μg/ml) were stopped after 2 h of incubation.

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Analysis of DNAM-1 expression

Healthy donor-derived PBMCs were coincubated with target cells at a ratio of 10:1 as described above. Before analysis, cells were stained with a dead cell marker and a combination of anti-CD3, anti-CD14, anti-CD56, and anti-DNAM-1 mAbs for 15 min on ice. GolgiPlug and an anti-CD107a mAbs were used for the analysis of DNAM-1 expression on activated cells as described for the CD107a assay above. The relative expression (MRFI) was calculated as the mean fluorescence intensity of DNAM-1 on NK cells coincubated with target cells divided by the mean fluorescence intensity of DNAM-1 on NK cells incubated without target cells.

Statistics

Statistical analyses were performed with GraphPad Prism (GraphPad Software) using the Wilcoxon and the Mann-Whitney tests for paired and unpaired groups, respectively, and one-way ANOVA with Dunn’s multiple comparison for multiple comparison analyses. Correlation analysis was performed using Spearman’s correlation test.

Results

Perturbed NK cell receptor repertoires and altered subset frequencies in peritoneal effusions of patients with ovarian carcinoma

We performed a high-resolution phenotypic analysis of NK cells from peritoneal effusions and peripheral blood of patients with ovarian carcinoma. Tumor-associated NK cells differed substantially from autologous NK cells in peripheral blood and NK cells from sex- and age-matched healthy controls. First, we observed an increased proportion of CD56bright NK cells constituting on average 32% of all NK cells in the peritoneal effusions, compared with ~10% in peripheral blood (Fig. 1, A and B) (28). Containing with CD16, NKG2A, KIRs, CD57, and LIRLR-B1 confirmed a classical phenotype of the CD56bright NK cells (Fig. 1A) (3), Second,
FIGURE 1. Increased proportion of CD56 brightly NK cells and perturbed expression of NK cell receptors on CD56 dim NK cells in the tumor environment. A, Representative dot plot diagrams of PBLs from a healthy donor (HD; left), as well as of PBLs (middle) and autologous tumor-associated lymphocytes (TAL) in peritoneal effusion (right) from an ovarian carcinoma patient. The histograms show expression of markers used to define the two NK cell subsets. B, Proportion of CD56 brightly NK cells in total NK cells in PBLs from HDs (○; n = 6), PBL (▲; n = 11) and TAL in patients (▼; n = 11). C, Representative histograms for the expression of activating and costimulatory NK cell receptors on CD56 dim NK cells in PBLs (dashed line) and peritoneal effusions (line) compared with isotype-matched control mAb (filled). D, Expression of activating and costimulatory NK cell receptors on CD56 dim NK cells in PBLs from HDs (○; n = 6), PBL (▲; n = 11) and TAL (▼; n = 11) from patients. Lines indicate mean fluorescence intensity for each receptor. *, p < 0.05; ***, p < 0.001; ****, p < 0.0001.
tumor-associated NK cells displayed multiple alterations in the receptor repertoires of both the CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK cell subsets (Fig. 1, C and D, and supplemental Fig. 1, respectively). Analysis of the CD56\textsuperscript{dim} NK cell subset revealed that expression of the activating receptor DNAM-1 was significantly lower on tumor-associated NK cells compared with NK cells isolated from peripheral blood of patients and healthy donors (Fig. 1D). Similarly, the coactivating receptor 2B4 (CD244) was relatively lower on tumor-associated NK cells (Fig. 1D). Furthermore, the CD16 receptor, primarily expressed on CD56\textsuperscript{dim} NK cells, was significantly decreased on the tumor-associated CD56\textsuperscript{dim} NK cells (Fig. 1D). In contrast, the expression of NKp46 and NKG2D was slightly higher on NK cells within the tumor environment, whereas the expression of NKp30 was unaltered compared with NK cells in peripheral blood (Fig. 1D). Analysis of the CD56\textsuperscript{bright} NK cell subset revealed similar receptor alterations as for CD56\textsuperscript{dim} NK cells (supplemental Fig. 1).

These results demonstrate that NK cells in the tumor environment display altered proportions of the CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cell subsets compared with peripheral blood and perturbed expression of activating NK cell receptors, including significant down-modulation of DNAM-1, 2B4, and CD16.

Changes in 2B4 and NKG2D expression on CD56\textsuperscript{dim} NK cells correlate with increased proportion of CD56\textsuperscript{bright} NK cells in the peritoneal effusion

The variable degrees of receptor modification among patients prompted us to examine whether there was any correlation between the increased proportion of CD56\textsuperscript{bright} NK cells and the observed down-regulation of DNAM-1, 2B4, and CD16. Interestingly, the increased relative proportion of CD56\textsuperscript{bright} NK cells in the peritoneal effusion correlated to the loss of expression of the 2B4 receptor ($p < 0.05$). An opposite tendency was observed for the expression of NKG2D ($p = 0.07$), whereas no correlation was observed for none of the other receptors studied, including DNAM-1, NKp30, NKp46, and CD16 (Fig. 2). This observation indicates that the coordinated increase in CD56\textsuperscript{bright} NK cells and changes in 2B4 and NKG2D are related and may depend on environmental factors. However, despite the fact that several cytokines, including IL-2, IL-10, and IFN-\gamma, were increased in peritoneal effusions compared with autologous plasma, exposure of peripheral blood-derived NK cells to peritoneal effusion from patients with high proportions of CD56\textsuperscript{bright} NK cells did not mimic the observed changes in receptor expression (data not shown).

Importantly, neither of these observations could explain the reduced expression of the DNAM-1 receptor. Since this receptor is of fundamental importance for the recognition and killing of several human tumors, including ovarian carcinoma (15–17, 29), we next investigated the mechanism behind the modulation of DNAM-1 receptor expression in the tumor environment.

Loss of DNAM-1 expression upon interaction with CD155-expressing freshly isolated ovarian carcinoma cells

Receptor-ligand interactions have been shown to down-modulate surface expression of several NK cell receptors, including NKG2D (30, 31). Therefore, we studied if there was a correlation between the reduced levels of DNAM-1 expression on NK cells in peritoneal effusions with the expression of CD155 on autologous ovarian carcinoma cells. This analysis revealed a significant inverse correlation between the relative reduction of DNAM-1 expression on tumor-associated CD56\textsuperscript{dim} NK cells and the expression of CD155 on carcinoma cells (Fig. 3A). To more directly assess the role of
CD155, we used a recently described system in which S2 insect cells are transfected with ligands of human NK cell receptors ligands (27). Coincubation of peripheral blood NK cells from healthy donors with S2 cells expressing CD155 induced specific down-modulation of DNAM-1 (Fig. 3B). Importantly, no reduction of DNAM-1 expression was observed upon interaction with S2 cells expressing the NKG2D ligand ULBP1 (Fig. 3B).

To investigate the consequences of receptor ligation in a more physiological setting, we used a panel of freshly isolated ovarian carcinoma cells and tumor cell lines, all expressing CD155 (supplemental Fig. 2). In agreement with our previous report, all tested tumor samples were negative for Nectin-2 (data not shown and Ref. 15). Coincubation of NK cells with the ovarian carcinoma cell lines CaOV-4 and Skov3 as well as the freshly isolated ovarian carcinoma cells resulted in a reduction of DNAM-1 expression (Fig. 3C). Similarly, DNAM-1 was significantly down-modulated upon coincubation with K562 cells that also express CD155. In contrast, coincubation with the NK cell-resistant P815 cell line and the NK-sensitive 721.221 cell line, both lacking CD155 expression, did not induce DNAM-1 down-modulation, regardless of the degree of NK cell activation (Fig. 3C and data not shown). Kinetic experiments demonstrated a gradual reduction of DNAM-1 expression, occurring over 12 h during coincubation with fresh tumor cells (Fig. 3D).

To assess the role of soluble factors in the modulation of DNAM-1 receptor expression, we next coincubated healthy donor-derived NK cells with target cells in the presence of a transwell membrane abrogating physical interactions between DNAM-1 and CD155. DNAM-1 expression remained intact when physical interactions were prevented, whereas reduced DNAM-1 expression was observed when target cells expressing CD155 were allowed to interact with the NK cells (Fig. 4A). Furthermore, maintaining NK cells in peritoneal effusions for 36 h did not affect surface expression of DNAM-1, excluding a role for soluble factors including DNAM-1 ligands and/or cytokines in the down-modulation of this receptor within this time frame (Fig. 4B).

Hence, these data suggests that a physical interaction between CD155 and DNAM-1 is required to induce a loss of DNAM-1 receptor expression.

**Impaired activation of tumor-associated NK cells upon specific stimulation via the DNAM-1 receptor**

To explore the functional responsiveness of CD56dim tumor-associated NK cells, we monitored the cell surface expression of CD107a as a surrogate marker for degranulation (32). The overall responsiveness of patient-derived CD56dim NK cells following

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**FIGURE 3.** Loss of DNAM-1 on NK cells following interaction with CD155-expressing tumor cells. A. Relative expression of DNAM-1 on peritoneal effusion-derived CD56dim NK cells compared with autologous peripheral blood-derived CD56dim NK cells was plotted against the geometric mean relative expression intensity (GMRFI) of CD155 on ovarian carcinoma cells. DNAM-1 expression was measured on CD56dim NK cells following coincubation of healthy donor-derived PBMCs with the indicated tumor targets. B. Relative expression of DNAM-1 on NK cells (n = 4) after coincubation for 4 h with S2 cells transfected with the indicated NK receptor ligands. C. Relative expression of DNAM-1 on NK cells (n = 5) after coincubation for 12 h with the indicated tumor targets. D. Relative expression of DNAM-1 on NK cells (n = 3) at several time points after coincubation with fresh ovarian carcinoma cells. P815 cells and K562 cells were used as negative and positive controls, respectively. Results are representative of two independent experiments. Columns show relative expression of DNAM-1; bars, SD. *, p < 0.05; ***, p < 0.01; ***, p < 0.001.
DNAM-1 is an activating NK cell receptor recently demonstrated to play an important role in tumor immune surveillance. Here we describe that receptor engagement attenuates DNAM-1 expression on NK cells, leading to hyporesponsiveness. These results suggest that chronic receptor-ligand interactions may cause loss of DNAM-1 expression on NK cells in the tumor environment, thereby contributing to poor NK cell-mediated elimination of ovarian carcinoma cells and possibly also other tumors expressing DNAM-1 ligands.

The importance of DNAM-1 on both T cells and NK cells was recently highlighted in a DNAM-1 knockout mouse model (12, 34). Data revealed a central role for DNAM-1 in costimulation of CD8 T cells upon recognition of nonprofessional APCs (34). DNAM-1 was also shown to be critical in the recognition of tumor cell lines expressing DNAM-1 ligands. Additional evidence for

Discussion

DNAM-1-mediated loss of DNAM-1 impaired tumor targeting

A redirected lysis assay was used to assess the consequences of reduced CD16 expression on tumor-associated NK cells. Stimulation of CD16 on CD56dim NK cells from peripheral blood effusions led to significantly lower degranulation and cytokine production compared with triggering of CD16 on the corresponding subset in peripheral blood (Fig. 6, A and B, and data not shown). Next, NK cell-mediated ADCC was evaluated against tumor cells coated with trastuzumab (Herceptin), a mAb specific for human epidermal growth factor receptor 2 (HER-2/neu) that is expressed by metastatic ovarian carcinoma cells (33). The response of tumor-associated NK cells upon ADCC to Ab-coated autologous ovarian carcinoma cells was abrogated compared with NK cells isolated from peripheral blood from the same patients (Fig. 6C). Rituximab, another humanized mAb used in clinical therapy targeting CD20-expressing cells, was used as a negative control Ab and resulted in low responses by both NK cell populations. Thus, tumor-associated NK cells display an impaired activation when stimulated via the CD16 receptor pathway, leading to an abrogated capacity for ADCC against autologous ovarian carcinoma cells.

![Diagram of DNAM-1 expression](http://www.jimmunol.org/)
DNAM-1-dependent rejection of tumors was provided by increased formation of ligand-expressing fibrosarcomas and papillomas after treatment of mice with the carcinogens methylcholanthrene and 7,12-dimethylbenz[a]anthracene, respectively (12). These studies provide the first direct evidence for a central role for DNAM-1 in tumor immune surveillance.

DNAM-1 is important for NK cell-mediated recognition of several human tumors, including neuroblastoma, myeloma, and Ewing sarcoma (16, 17, 29, 35). We have previously shown that the DNAM-1/CD155 interaction is crucial for the recognition of freshly isolated ovarian carcinoma by allogeneic NK cells (15). Thus, loss of DNAM-1 expression on NK cells may explain the inability of patient-derived NK cells to kill autologous tumors (24).

Suppression of NK cell function in cancer patients has been associated with reduced expression of activating NK cell receptors, but the mechanisms for receptor modulations are not fully understood (36–38). Mechanisms such as shedding of ligands, chronic ligand exposure, and trogocytosis have been described (30, 31, 39, 40). Interestingly, CD96, a NK cell receptor that like DNAM-1 binds CD155, was shown to be down-regulated upon ligand engagement (41). Since ovarian carcinoma cells constitutively express CD155 (15), we speculated that similar mechanisms could be responsible for the down-modulation of DNAM-1 on tumor-associated NK cells. Indeed, we were able to demonstrate that peripheral blood NK cells lost DNAM-1 expression within hours of exposure to CD155-expressing targets. Down-modulation of

**FIGURE 5.** Impaired activation of tumor-associated NK cells in response to specific stimulation via DNAM-1. The expression of CD107a was measured on CD56dim NK cells isolated from PBLs of healthy donors (PB-NK (HDs); n = 4; dashed bars) and ovarian carcinoma patients (PB-NK (Patients); n = 6; open bars) as well as from peritoneal effusions (TA-NK (Patients); n = 8; filled bars). A, Representative FACS plots showing CD107a expression after stimulation with PMA and ionomycin or K562 cells. B, CD107a expression after stimulation with PMA and ionomycin or K562 cells. C, Representative FACS plots showing the CD107a expression after stimulation with P815 cells coated with a combination of agonistic mAbs specific for either DNAM-1 and 2B4 or NKGD2 and Nkp46. D, CD107a expression after stimulation with P815 cells coated with indicated combination of agonistic mAbs. IgG1 isotype control mAbs were used as negative control stimuli. Columns show mean of CD107a expression (%); bars, SD. *, p < 0.05; **, p < 0.01; ***, p < 0.001. E, Histograms showing the killing of freshly isolated ovarian carcinoma cells by autologous NK cells isolated from OC93. One representative experiment of two is shown.
DNAM-1 was dependent on physical contact with target cells expressing CD155 since no change in DNAM-1 expression was observed when effectors and targets were separated in transwell experiments or when NK cells were exposed to peritoneal effusions. Interestingly, we found an inverse correlation between the expression of CD155 on ovarian carcinoma cells and the expression of DNAM-1 on autologous tumor-associated NK cells, supporting the notion that these events take place in vivo.

Receptor-ligand interactions occurring during target recognition could not explain the loss of 2B4 expression since the ligand for 2B4, CD48, is not expressed by ovarian carcinoma cells. Furthermore, the requirement for physical contact between NK cells and target cells to down-regulate DNAM-1 expression, and transwell experiments revealed a requirement for physical contact between NK cells and target cells for the loss of DNAM-1 expression.

Increasing knowledge of the molecular specificities in NK cell-mediated tumor recognition provides new possibilities for developing more effective immunotherapeutic interventions (56). For efficient tumor rejection, strategies to circumvent the immunomodulatory effects of tumor environments are likely needed in combination with adoptive transfer of NK cells. A recent report has shown that sequential killing of multiple target cells (57) may be hampered through the loss of DNAM-1 expression following initial target cell contact. Repetitive adoptive transfer of DNAM-1-expressing NK cells may help to overcome the continuous down-regulation of DNAM-1 upon interaction with CD155-expressing tumor targets. Chimeric NKG2D receptors were up-regulated by tumor-associated NK cells upon specific ADCC by tumor-associated NK cells upon specific stimulation via the CD16 receptor. The expression of CD107a was measured on CD56<sup>bright</sup> NK cells isolated from PBL of healthy donors (PB-NK (HDs); n = 4; dashed bars) and ovarian carcinoma patients (PB-NK (Patients); n = 7; open bars) as well as from peritoneal effusions of patients (TA-NK (Patients); n = 10; filled bars). A, Representative FACS plots showing the CD107a expression after stimulation with P815 cells coated with agonistic CD16 mAb. B, CD107a expression after stimulation with P815 cells coated with agonistic CD16 mAb. IgG1 isotype control mAbs were used as negative control stimuli. C, CD107a expression on patient derived CD56<sup>dim</sup> NK cells (n = 6) after coincubation with trastuzumab-coated freshly isolated autologous ovarian carcinoma cells. The same target cells, coated with rituximab Ab targeting CD20 Ags, were used as negative control stimuli. Columns show mean of CD107a expression (%); bars, SD. *p < 0.05; **p < 0.01.
have been shown to enhance tumor targeting by CTLs (58). Similar approaches based on effecter cells that stably express chimeric DNAM-1 receptors could theoretically also enable effective tumor rejection by NK cells.

The efficacy of mAbs for the treatment of malignancies such as lymphoma and breast cancer is well established (59). Since metastatic ovarian carcinoma cells uniformly express the tumor Ag Her2/neu, they could serve as targets for the humanized mAb trastuzumab (33). Apart from changes in DNAM-1 expression, our phenotypic analysis demonstrated a dramatic loss of CD16 expression, which severely impaired tumor-associated NK cell ADCC toward trastuzumab-coated fresh ovarian carcinoma cells. In contrast, autologous peripheral blood NK cells displayed robust activation upon coculture with trastuzumab-coated targets. It has previously been reported that the loss of the signal transducing molecules FcγRIa and CD3-ζ in tumor-associated lymphocytes of patients with ovarian carcinoma led to reduced expression of CD16 and depressed the proliferative response to CD16 stimulation (60). Moreover, monocyte-derived macrophages from both peripheral blood and peritoneal effusions of ovarian cancer patients had less ADCC activity than did the corresponding cells from normal donors (61). These results indicate that targeting of metastatic ovarian carcinoma with mAbs, via NK cell-mediated ADCC, may be less effective than anticipated.

In conclusion, we have demonstrated that the loss of DNAM-1 expression on tumor-associated NK cells results in impaired NK cell activation and that loss of CD16 abrogates the killing of trastuzumab-coated autologous ovarian carcinoma cells. Moreover, we provide evidence for the contribution of DNAM-1/CD155 interactions to the reduction of DNAM-1 expression, suggesting that chronic receptor-ligand interactions in the tumor environment may induce loss of DNAM-1 on tumor-associated NK cells. These results may have implications for the design of future protocols of adoptive NK cell- and Ab-based immunotherapies for ovarian carcinoma and possibly other human tumors.

Disclosures

The authors have no financial interests of conflict.

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